Medical Library

INDEX NUMBER

JAN 3 1946

VOL. 30 DECEMBER, 1945 No. 12

The Journal of Laboratory and Clinical Medicine

Clinical and Experimental

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Vol. 30, No. 12, December, 1945. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis (3), Mo. Subscription Price: United States, its Possessions, Pan-American Countries, $8.50 per year; Canada, $9.50; Foreign, $9.50. Entered as Second-Class Matter October 8, 1915, at the Post Office, at St. Louis, Missouri, under Act of March 3, 1879. Printed in the U. S. A.

A COMPARISON OF THE RESULTS OBTAINED WITH THE HANGER CEPHALIN-CHOLESTEROL FLOCCULATION TEST AND THE MACLAGAN THYMOL TURBIDITY TEST IN PATIENTS WITH LIVER DISEASE

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With the Technical Assistance of Violet Hawkinson and Margaret Giebenhain

MACLAGAN has recently reported a study of the behavior of various human sera with a buffered thymol solution.[1, 2] This study was based upon the concept that globulins are precipitated more or less readily by phenolic compounds. Maclagan referred specifically to phenol as in the Pandy test and to cholesterol in the cephalin-cholesterol flocculation test of Hanger.[3] It was found that other phenolic compounds did, in fact, precipitate globulin. Thymol was the most satisfactory of any of a number tried. Employing a saturated aqueous solution of thymol buffered with barbitone and sodium barbitone to a pH of 7.8, Maclagan found that essentially the same results were obtained with various normal and pathologic sera as with the colloidal gold test, in which the detection of increased amounts of globulin depends upon precipitation of the gold. The turbidity occurring in abnormal sera when brought together with the buffered thymol solution was shown to be due to precipitation of a complex containing protein, thymol, cholesterol, and a phospholipid of lecithin type. Maclagan measured the degree of turbidity by comparing the solution after one-half hour with the formazin standards devised by Kingsbury and associates[4] and used for many years in the rough quantitative determination of protein in the urine. Using this method, Maclagan found that normal sera ranged from 0 to 4 units.

$$\text{Number of units} = \frac{\text{Standard tube reading} \times \text{final dilution of serum}}{600}$$, the standard dilution being 1 to 60 (0.05 c.c. of serum and 3.0 c.c. of the buffered thymol solution)

In 120 of 130 cases of hepatitis (stage of disease not given), significantly positive values above 4 units were observed. The same was true in all of thirteen cases of cirrhosis of the liver. However, in thirty-seven cases of jaundice due to extrahepatic biliary obstruction, only three exhibited values above 4 units; these were in the 5 to 7 range.

Assuming that Maclagan's thymol turbidity test was to provide essentially the same information as the cephalin-cholesterol flocculation test of Hanger, it would offer a number of important advantages, including (1) an easily prepared stable solution containing weighed amounts of pure chemicals, (2) completion in one-half hour instead of forty-eight hours, and (3) a simple method

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Aided by the Josiah Macy, Jr., Foundation, New York City.

Received for publication, Sept. 24, 1945.

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of estimating the degree of positivity. Although Maclagan implied that the basis of the thymol turbidity test is identical with that of the cephalin-cholesterol flocculation, he did not present any data to reveal whether the results with the two methods are equivalent.

The purpose of the present communication is to present data obtained by comparing the cephalin-cholesterol flocculation test and the thymol turbidity test as applied to various human sera. The cephalin-cholesterol flocculation test was performed according to Hanger's method, employing Difco* antigen. In carrying out the thymol turbidity test, 0.1 c.c. of serum was diluted to 6 c.c. with the thymol buffer solution, rather than 0.05 to 3, as according to Maclagan; in other respects the procedure was identical. The turbidity of the final solution is estimated by comparison with Kingsbury's standards.[4] These consist of ten tubes with degrees of turbidity corresponding to a range of from 10 to 100 mg. Prepared tubes obtained on the market† have been found to be satisfactory except that the only concentrations included above 50 mg. per cent are the 75 and 100 mg. per cent tubes. This requires that sera producing turbidity greater than that of the 50 mg. per cent tube must be diluted further.

The data which will be considered are based upon 252 Hanger and Maclagan tests carried out on samples of blood serum from 145 individuals and may be divided as follows:

GROUP	NUMBER OF INDIVIDUALS	NUMBER OF TESTS
I—Presumably normal adults	31	31
II—Hepatitis	60	154
III—Cirrhosis and subacute atrophy	13	19
IV—Extrahepatic biliary obstruction		
Calculous	5	5
Cancer	6	8
Stricture	1	1
V—Hepatic carcinomatosis	4	4
VI—Inoculation malaria	6	10
VII—Miscellaneous	18	20

In this study the diagnosis was based on clinical features alone in the patients in Groups I, II, and VI, while in those included in Groups III, IV, and V the diagnosis was established either at operation or autopsy, or by means of liver biopsy.

RESULTS

The data are shown in Tables I to VII, inclusive. It is seen that, in the main, there is a distinct correlation between the two tests. If one accepts Maclagan's value of 4 units for the upper limit of a negative test and, similarly, for the Hanger test a value of a trace for twenty-four hours or 1 plus for forty-eight hours, twenty-seven instances are found in the present data in which one test was positive and the other negative. In other words, there was agreement in 225 of 252 tests, or 89.3 per cent, on the arbitrary basis just mentioned. The twenty-seven instances of disagreement are brought together in Table VIII. It is seen that the majority of these are borderline. In the patients with hepatitis the discrepancies noted were of little moment as they were associated with transitions in the course of the disease either toward recovery or relapse, usually the former (see Table II). In each instance the trend of each test and the information gained were essentially the same.

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More serious discrepancies are encountered in Groups III, IV, and V, as noted in Table VIII. In both cases of subacute atrophy distinctly positive Hanger tests with negative thymol turbidity were exhibited. A discrepancy was noted in but one of the eleven cases of cirrhosis, and it is perhaps of some interest that this was the only example of "alcoholic" cirrhosis in the group. The other cases were of unknown etiology or represented transitions from infectious hepatitis, cases which have been referred to as "cholangiolitic cirrhosis."[5] As noted in Table III there was also one example of hemochromatosis and one which was first thought to represent the so-called xanthomatous biliary cirrhosis; however, at autopsy the latter was shown to be only an ordinary portal cirrhosis.[6]

But ten cases of proved cancer are represented in the series and it is interesting that there were varying discrepancies in six of these, as given in Table VIII. A rather remarkable reversal in Case 6 (Group IV) occurred within a period of three weeks. The significance of this is not at all clear.

Among the 89 per cent in whom both tests were positive, according to the arbitrary division referred to in the foregoing, many instances of poor quantitative correlation were encountered; for example, sera exhibiting a 3 plus, 4 plus Hanger test with but 5 or 6 units of thymol turbidity. Conversely there are a number of examples of a 2 plus, 3 plus Hanger test with 10 units or more of thymol turbidity. Case 14 in Group II, hepatitis (Table II), illustrates the type of variation in relative discrepancy that may be encountered especially when serial tests are being performed. The first result in this case was a Hanger test of 1 plus, 2 plus, and a Maclagan test of 10 units; sixteen days later the Hanger test was 2 plus, 3 plus and the Maclagan test was 8 units. In Cases 55 and 56, in the same group, it is seen that at certain periods Hanger tests of 3 plus, 4 plus and Maclagan tests of 5 units were observed. Other and similar lack of quantitative correlation is frequently observed in the tables.

TABLE I. GROUP I—PRESUMABLY NORMAL ADULTS

CASE	HANGER TEST		MACLAGAN TEST
	24 HR.	48 HR.	IN UNITS
1	0	0	2
2	0	0	2
3	0	0	2
4	0	0	3
5	0	0	2
6	0	0	3
7	0	0	2
8	0	0	4
9	0	0	3
10	0	0	3
11	0	0	3
12	0	0	1
13	0	0	1
14	0	0	1
15	0	0	1
16	0	0	1
17	0	0	1
18	0	0	1
19	0	Trace	1
20	0	0	1
21	0	0	1
22	0	0	1
23	0	0	1
24	0	0	1
25	0	0	1
26	0	0	1
27	0	0	1
28	0	0	1
29	0	0	1
30	0	0	1
31	0	0	1

TABLE II. GROUP II—HEPATITIS

CASE	DATE	HANGER 24 HR.	HANGER 48 HR.	MACLAGAN TEST IN UNITS
1	2/16	0	1+	4
	2/17	1+	1+	5
	2/25	1+	2+	6
	3/ 3	1+	2+	6
	3/ 5	2+	3+	8
	3/ 7	2+	3+	9
	3/19	2+	2+	7
2	2/20	0	0	1
	2/27	0	0	4
	3/3	0	0	2
3	2/19	0	0	3
	2/21	0	0	4
	3/17	0	0	2
4	2/16	0	1+	5
	3/13	0	0	2
	3/17	0	0	3
	3/23	0	0	2
5	3/16	0	0	4
	3/20	1+	1+	6
	3/29	0	1+	5
	4/2	0	0	4
6	2/16	1+	1+	6
	2/19	1+	2+	6
	3/10	0	1+	5
	3/15	0	0	4
	3/29	0	0	3
7	3/20	0	0	3
8	2/22	0		4
	3/31	0	0	3
9	3/16	1+	2+	7
	3/29	1+	2+	6
	4/4	1+	1+	5
	4/9	0	1+	5
10	2/22	0		4
	3/5	0		2
11	3/29	0	0	3
12	2/22	0		3
	3/3	0		2
13	2/15	1+	1+	5
	2/26	1+	1+	4
	3/5	0	0	3
	3/14	0	1+	4
	3/16	1+	1+	5
	3/19	0	1+	5
14	2/5	1+	2+	10
	2/10	2+	3+	10
	2/21	2+	3+	8
	2/23	1+	2+	8
	2/27	1+	2+	7
	3/3	2+	2+	7
	3/9	2+	3+	7
	3/16	2+	3+	8
15	3/8	2+	3+	9
	3/10	2+	3+	10
	3/14	2+	2+	7
	3/19	0	0	4
	3/28	0	0	3
16	2/16	3+	4+	10
	2/19	3+	4+	12
	2/21	2+	3+	10
	2/26	2+	3+	7
17	2/16	3+	4+	8
	2/21	2+	3+	9
	3/3	3+	3+	9
	3/19	1+	1+	6
18	3/1	2+	3+	8
	3/10	3+	4+	10
	4/3	2+	2+	7
	4/9	1+	1+	5
19	2/26	0		4
	2/28	0		2
20	3/3	0	0	3
21	2/22	1+	1+	5
	2/27	1+	1+	5
	3/3	1+	1+	4
22	3/20	0	0	3
	3/21	0	0	2
23	2/20	0		3
24	2/21	0		2
25	2/15	0	0	3
	2/17	0	0	2
26	2/26	2+	3+	8
	3/6	1+	1+	7
	3/12	0	0	4
	3/17	0	0	3
27	2/20	3+		8
	2/27	1+	2+	7
	3/6	1+	2+	6
	3/10	0	0	3
28	2/26	1+	1+	3
	3/13	0	0	3
	3/15	0	0	2
29	3/6	0		3
30	3/9	0	0	3
31	4/9	0	0	4
	4/12	0	0	3
32	3/6	1+	2+	5
	3/15	1+	2+	7
	3/17	2+	3+	8
33	3/8	0	0	2
34	2/26	0		4
	3/3	0		2
35	3/5	0		3
36	3/1	0		3
37	2/22	1+	1+	6
	3/1	0	0	4
	3/3	0	1+	5
	3/5	1+	1+	5
	3/19	0	0	3
38	2/22	0		2

CASE	DATE	HANGER 24 HR.	HANGER 48 HR.	MACLAGAN TEST IN UNITS
39	2/19	0		3
40	2/28	0		3
41	2/22	0		3
42	6/13	3+	4+	10
	6/20	2+	3+	7
	7/6	2+	3+	12
43	6/20	2+	3+	6
	6/27	2+	3+	7
44	7/5	3+	3+	16
	7/11	3+	4+	18
45	6/27	2+	3+	16
		2+	2+	20
		1+	2+	16
		1+	2+	15
46	6/2	0	Trace	1
47	3/12	3+	4+	6
	3/16	3+	4+	10
		2+	2+	7
		1+	2+	5
		0	1+	4
48	1/25	2+	3+	8
		3+	4+	9
49	3/8	3+	4+	9
	3/19	2+	3+	5
	3/21	3+	4+	7
	4/23	0	0	2
50	6/2	1+	2+	4
51	6/20	3+	4+	8
52	6/11	2+	2+	7
	6/18	1+	2+	5
53	3/16	3+	4+	7
54	3/7	1+	2+	6
55	3/8	3+	4+	5
	3/14	2+	3+	2
	3/16	3+	4+	3
	4/23	0	1	2
56	4/10	3+	4+	5
	4/12	2+	3+	5
		0	Trace	2
57	4/21	2+	3+	8
	4/25	2+	2+	5
58	4/10	2+	3+	5
59	5/16	1+	1+	2
60*	4/10	0	Trace	4

*Postarsphenamine.

TABLE III. GROUP III—CIRRHOSIS AND SUBACUTE ATROPHY

CASE	DATE	HANGER TEST 24 HR.	HANGER TEST 48 HR.	MACLAGAN TEST IN UNITS
1*		3+	4+	10
2†	5/16	Trace	1+	1
	5/31	0	1+	1
3	5/29	3+	4+	12
4	6/11	0	1+	3
5	5/18	3+	4+	12
	5/23	3+	4+	12
	5/25	3+	4+	12
	5/28	3+	4+	14
	7/11	2+	3+	24
	8/24			30
6	6/12	1+	2+	3
7	1/29	0	1+	5
8	2/9	3+	4+	7
9	7/25	1+	1+	8
10	8/17	2+	3+	2
11	8/20	4+	4+	16
12‡	6/9	2+	3+	1
13‡	4/19	3+	3+	2

*With hypercholesterolemia and xanthomata; autopsy revealed Laennec cirrhosis without evidence of so-called xanthomatous biliary cirrhosis.[6]

†Hemochromatosis.

‡Subacute atrophy.

TABLE IV. GROUP IV—EXTRAHEPATIC BILIARY OBSTRUCTION

	CASE	DATE	HANGER TEST 24 HR.	HANGER TEST 48 HR.	MACLAGAN TEST IN UNITS
Cancer	1	1/8	0	0	3
	2	5/16	1+	2+	1
	3	6/6	0	0	1
	4	1/23	Trace	1+	2
	5	1/25	2+	3+	7
	6	6/22	0	Trace	2
		7/2	0	1+	5
		7/24	3+	4+	1
Calculous	7	5/2	Trace	Trace	1
	8	3/17	0	0	3
	9	7/12	Trace	1+	1
	10	7/2	0	Trace	1
	11	7/18	0	0	1
Stricture	12	5/30	0	0	1

TABLE V. GROUP V—HEPATIC CARCINOMATOSIS

CASE	DATE	HANGER TEST 24 HR.	HANGER TEST 48 HR.	MACLAGAN TEST IN UNITS
1	1/30	1+	2+	3
2	3/16	2+	3+	2
3	3/10	1+	2+	3
4	8/6	1+	2+	2

TABLE VI. GROUP VI—INOCULATION MALARIA

CASE	CHILLS	DATE	HANGER TEST 24 HR.	HANGER TEST 48 HR.	MACLAGAN TEST IN UNITS
1	Before	1/27	0	0	2
	During	2/20	3+	4+	5
2	Before	1/2	0	0	2
	During	1/19	2+	3+	7
3	During	1/11	3+	4+	8
	During	1/24	3+	4+	14
4	During	12/23	3+	4+	7
	After	1/23	1+	2+	2
5	After	1/23	0	0	2
6	After	1/30	0	0	2

DISCUSSION

The present results indicate quite clearly that the underlying basis of the Hanger and Maclagan tests is not identical. Although an agreement was noted in 89 per cent of the tests, it is recognized that discrepancies in degree of positivity were frequent. Such discrepancies preclude any general substitution of the Maclagan for the Hanger test, at least in so far as routine use for differential diagnosis is concerned. The present study indicates that the Maclagan test is probably more often negative than the Hanger test in patients with serious diffuse liver disease, such as subacute yellow atrophy and certain forms of cirrhosis. Further study may reveal that certain forms of cirrhosis are more likely to exhibit negative thymol turbidity and positive cephalin flocculation. It has already been noted that the only case in the present series in which this was observed was one of alcoholic (fatty) cirrhosis. The present data suggest that the thymol turbidity test is more often negative and hence less likely to offer confusion in diagnosis in cases of hepatic or biliary tract cancer. In so far as the hepatitis problem is concerned, the present data reveal

TABLE VII. GROUP VII—MISCELLANEOUS

CASE	CONDITION	DATE	HANGER TEST 24 HR.	HANGER TEST 48 HR.	MACLAGAN TEST IN UNITS
1	Infectious mononucleosis	3/16	1+	2+	1
		3/20	2+	3+	6
2	Hepatic vein thrombosis (polycythemia)	1/23	1+	2+	2
3	Pneumonia and empyema	1/22	Trace	1+	2
4	Cholelithiasis	3/7	0	0	2
5	Tuberculous peritonitis?	3/7	Trace	1+	3
6	Pneumococcus pneumonia with jaundice and numerous spider nevi	4/20	0	0	4
		5/2	0	0	2
7	Hemolytic jaundice	6/15	2+	3+	2
8	Rheumatoid arthritis	5/26	0	0	3
9	Hypochromic anemia	6/18	1+	2+	3
10	Inferior vena cava thrombosis (metastatic cancer?)	6/18	2+	3+	10
11	Hypercholesterolemia	6/18	0	0	1
12	Hemolytic jaundice	1/30	1+	1+	3
13	Pancreatic adenoma	7/24	0	0	1
14	T-tube drainage of common duct	8/6	0	0	2
15	Cholelithiasis	8/22	0	Trace	3
16	C.N.S. syphilis	1/23	Trace	1+	2
17	C.N.S. syphilis	3/9	0	1+	2
18	C.N.S. syphilis	1/16	0	Trace	1

TABLE VIII. DISAGREEMENT BETWEEN TESTS

	CASE	HANGER TEST 24 HR.	HANGER TEST 48 HR.	MACLAGAN TEST IN UNITS
Group II—Hepatitis	4	0	1+	5
	5	0	1+	5
	6	0	1+	5
	9	0	1+	5
	13	0	1+	5
	21	1+	1+	4
	28	1+	1+	3
	37	0	1+	5
	50	1+	2+	4
	59	1+	1+	2
Group III—Cirrhosis	6	1+	2+	3
	7	0	1+	5
	10	2+	3+	2
Subacute atrophy	12	2+	3+	1
	13	3+	3+	2
Group IV—Biliary obstruction	2	1+	2+	1
(cancer)	6	0	1+	5
		3+	4+	1
Group V—Hepatic carcinomatosis	1	1+	2+	3
	2	2+	3+	2
	3	1+	2+	3
	4	1+	2+	2
Group VI—Inoculation malaria (after fever)	4	1+	2+	2
Group VII—Miscellaneous				
Infectious mononucleosis	1	1+	2+	1
Hepatic vein thrombosis	2	1+	2+	2
Hemolytic jaundice	7	2+	3+	2
Hypochromic anemia	9	1+	2+	2
Hemolytic jaundice	12	1+	1+	3

that the Maclagan test is fully as useful and reliable as the Hanger test once the disease is well established and during its defervescence. We have not had opportunity to compare the tests during the preicteric or prodromal stage. It is quite possible that the Hanger test may become positive earlier than the Maclagan, especially if the present results in the two cases of subacute atrophy can be correlated with the evidence of early diffuse hepatocellular injury in infectious hepatitis.[7] This question should be studied at the earliest possible moment since the Maclagan test, if equally reliable in the early stage of the disease, is much easier to use on a large scale; for example, during an epidemic when extensive screening for early cases is much to be desired.

Because of its relative simplicity and rapidity, the Maclagan test can certainly be employed to good advantage as a routine procedure; the Hanger test may be reserved for cases in which the diagnosis is still in doubt, or where the result of the Maclagan test disagrees with other procedures such as the one-minute and total serum bilirubin,[8] the urine Ehrlich test for urobilinogen,[9] and the 5 mg. per kilogram forty-five minute bromsulfalein test.[10] The Maclagan test is also very helpful for serial usage where it has once been shown to be positive, as in cases of hepatitis. In other words, it has considerable value in following the course of the disease in any particular instance.

As noted at the outset, Maclagan believed that the thymol turbidity is due to a globulin-thymol-lipid complex and that the test gives essentially the same results as the colloidal gold procedure which has been used in the study of liver disease. The earlier studies of Hanger and his associates likewise implicated a globulin, that is, the gamma globulin, as the important factor in the cephalin-cholesterol flocculation.[11] Very recently, however, Hanger and co-workers[12] described evidence indicating that an alteration in character of the serum albumin must be present, together with an increased globulin, before flocculation will take place. The addition of normal albumin to sera high in globulin content was found to prevent flocculation. This may well constitute an essential difference between the mechanisms of the Hanger and Maclagan tests, a difference which would readily explain the discrepancies noted in the present study. Such a difference might also explain the positive Hanger and negative Maclagan tests exhibited by normal sera of dogs and other species.*

SUMMARY AND CONCLUSIONS

1. The Hanger cephalin-cholesterol flocculation test and the Maclagan thymol turbidity test have been compared on 252 blood sera from 145 individuals. Thirty-one of these subjects were believed to be normal, while sixty (154 tests) were suffering from infectious hepatitis. In accordance with the previous findings of Hanger and Maclagan, the values in the normals did not exceed a trace (24 hr.) or 1 plus (48 hr.) for the cephalin flocculation or 4 units for the thymol turbidity.

2. Employing this as an arbitrary dividing line between positive and negative, an agreement of 89.3 per cent was noted. The majority of the 10.7 per cent of discrepancies between positive and negative were borderline and, in the hepatitis group especially, were not serious. In the cases of cirrhosis, subacute atrophy, and cancer, however, several serious discrepancies were encountered. In addition, a number of marked discrepancies in degree of positivity within the 89 per cent of correlation was observed.

3. It is evident that the underlying mechanism of the two tests is not identical and that the Maclagan test cannot be used interchangeably with, or

*Personal communications from Dr. F. M. Hanger and Dr. S. Schwartz.

as a complete substitute for, the Hanger test. Because of its simplicity, stability of reagents, and rapidity, it can be used to advantage for serial observations where the diagnosis has been established, as, for example, in cases of infectious hepatitis. Information is needed as to the relative behavior of the tests in the preicteric or prodromal stage of this disease.

4. The Maclagan test can also be used to advantage in the differential diagnosis of jaundice or liver disease, as long as its limitations are recognized. The present study indicates that a strongly positive test is just as indicative of liver injury or disease as is the Hanger test and that it may prove to be more uniformly negative in cases of jaundice due to biliary tract cancer and in cases of hepatic cancer with or without jaundice. Conversely, it appears that the Hanger test is more often positive in cases of cirrhosis or subacute atrophy. Additional data are needed to determine this point and to reveal whether the thymol turbidity test is more likely to be negative in one type of cirrhosis than another.

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ANTIGENIC PROPERTIES OF VARIOUS LIPOIDS FROM BEEF HEART

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INTRODUCTION

ALL efforts to make the serodiagnosis of syphilis absolutely infallible and perfectly specific have failed up to the present time. Nearly forty years of research have made it clear that there is still a discrepancy between the different methods with regard to sensitivity and specificity. The Washington Serology Conference (1941) tried to evaluate the older and also some of the more recently published American methods. The findings were not uniform; Mahoney[1] attributed the discrepant results to "the different sensitivity of the tests; to the weakness in the test mechanics when the concentration of the reacting substance is in the region of the threshold of the test; to the presence of certain factors which have the power of determining the test or tests which may yield a positive reaction while others are negative." Not one of the thirty-one separate procedures was absolutely specific for syphilis.

Only Hinton flocculation and the Mazzini complement fixation procedures gave entirely negative results with sera from patients with malaria. But not one of the thirty tests was reliable when used with sera from patients with leprosy. Relatively, the best were the Mazzini complement fixation test (specificity 86.2 per cent) and the Boerner-Jones-Lukens simplified complement fixation test (83.7 per cent).

To the many recognized diseases of nonsyphilitic origin which sometimes give a positive syphilitic reaction, the following have recently been added: hyperproteinemia (Cardon and Atlas[2]) and immunization against various diseases (Arthur and Hale,[3] Kahn and associates,[4] Lubitz,[5] and Rein and Elsberg[6]). The question of the so-called false positive reaction is still under discussion and unsolved. Actually a false positive reaction does not exist. There is a positive reaction in patients without syphilis (nonspecific) and there is a positive reaction due to a technical error in a nonsyphilitic person. The latter will become negative when repeated; the former will stay positive. There is, therefore, abundant reason for making further attempts to reduce the number of nonsyphilitic positive reactions. From the various ways of approaching this problem, one seems to be an improvement of the antigens.

It is generally accepted that human serum contains more than one reagin. In an editorial, four types of reagins are described.

1. The flocculating factor present in minute traces in many human sera.

2. The "reagin" present in the occasional human serum in amounts sufficient to give a positive or doubtful test.

3. The "reagin" elaborated in the course of such nonsyphilitic diseases as leprosy, malaria, cowpox, and infectious mononucleosis.

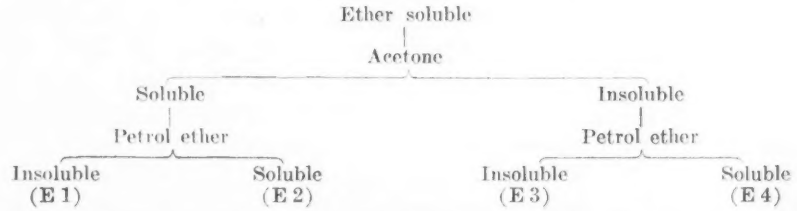
4. The "reagin" elaborated in the course of syphilitic infection. It can be added that the latter is composed of two kinds: (1) "reagins" against the

From the Mount Sinai Hospital.
Received for publication, Dec. 11, 1944.

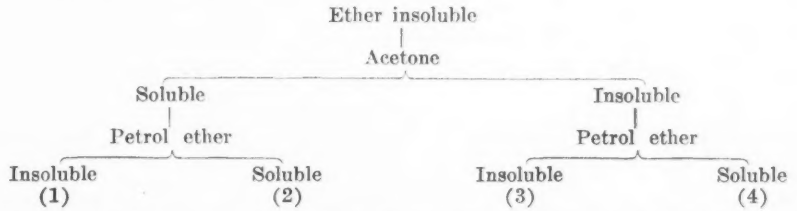
spirochetal lipoids as proved through the specific value of Gaehtgens'[8] and Klopstock's[9] spirochetal antigen and (2) the much more numerous nonspecific "reagins" which are detected with the commonly used beef heart antigens. Antigens are of very complex nature. To divide them into various parts and test their special action upon syphilitic and nonsyphilitic sera is the object of the following experiments.

TECHNICAL PROCEDURE

Experiment 1.—Twenty grams of dried beef heart powder* were extracted twice with 200 c.c. of anesthesia ether at 8° C. After evaporation of the ether, the residual lipoids were divided into acetone-soluble and acetone-insoluble fractions by dissolving them in 500 c.c. of acetone at 50° C. Each of these was extracted with petrol ether at room temperature, yielding an acetone-soluble-petrol ether-insoluble fraction (E 1), an acetone-soluble-petrol ether-soluble fraction (E 2), an acetone-insoluble-petrol ether-insoluble fraction (E 3), and an acetone-insoluble-petrol ether-soluble fraction (E 4).



Remaining beef heart powder after extraction with ether was then repeatedly and completely extracted with acetone at 50° C., and the acetone-soluble fraction was divided into a petrol ether-soluble fraction (2) and a petrol ether-insoluble fraction (1) by dissolving it in 200 c.c. petrol ether three times. The acetone-insoluble fraction was extracted three times with 200 c.c. petrol ether at room temperature (4) and the remaining acetone- and petrol ether-insoluble fraction was dissolved in absolute alcohol at 50° C. (3).



The final antigen was prepared in the proportion 1:200 with absolute alcohol with the addition of 2 c.c. of 1 per cent cholesterol in alcohol to each 100 c.c. absolute alcohol.

Experiment 2.—Without a previous ether extraction, 10 Gm. of dried beef heart powder* were extracted first with acetone and then with petrol ether to obtain an acetone-soluble-petrol ether-insoluble fraction (Ac 1), an acetone-soluble-petrol ether-soluble fraction (Ac 2), an acetone-insoluble-petrol ether-insoluble (Ac 3), and an acetone-insoluble-petrol ether-soluble fraction (Ac 4).

*Difco Laboratories, Inc., Detroit, Mich.

Another 10 Gm. of dried beef heart powder* were first extracted with petrol ether and then with acetone to get an acetone-soluble-petrol ether-insoluble fraction (Pe 1), an acetone-soluble-petrol ether-soluble fraction (Pe 2), an acetone-insoluble-petrol ether-insoluble fraction (Pe 3), and an acetone-insoluble-petrol ether-soluble fraction (Pe 4).

As with the antigens in Experiment 1, these were dissolved in absolute alcohol in the proportion 1:200 with the addition of 2 c.c. of 1 per cent cholesterol in alcohol to each 100 c.c. absolute alcohol.

Antigens for Experiments 1 and 2 were stored for three months at room temperature before being tested. The tests were performed using the active method (Hecht[10]) complement fixation technique.

The proper titrated amount of the antigen is measured into a wide-mouthed bottle. A 10 c.c. pipette is introduced into the bottom of the bottle and the saline solution allowed to flow out very slowly so that the antigen will be raised. Then the bottle is placed in the incubator (37° C.) without shaking so that the overlying alcoholic antigen can evaporate.

The next day, after the alcohol has evaporated, the bottle is well shaken, and the evaporated part is replaced by distilled water and the required amount of saline added to obtain the proper dilution. For example, if the dose of the antigen is 0.2 c.c. of 1:25 dilution in saline, 1.0 c.c. of antigen is taken and 10 c.c. of saline is placed under it; the next day distilled water is added to make 10 c.c.; saline is then added to make a total of 25 c.c.

The titration of the antigen must be made also with an alcohol-free emulsion prepared as described.

The use of an alcohol-free antigen has two objects and advantages; first, to eliminate the damaging influence of the alcohol upon the complement and, second, to avoid the nonspecific precipitation of an alcoholic antigen when mixed with sera from patients with pneumonia, tuberculosis, and other febrile conditions.

To determine the sensitivity of the antigens, pooled sera were used as shown in Table I.

TABLE I. POOLED POSITIVE AND NEGATIVE SERA MIXED IN VARIOUS AMOUNTS TO OBTAIN A MEDIUM POSITIVE SERUM

SERUM (C.C.)		KLINE TEST		
POS. (C.C.)	NEG.	DIAG.	CONTROL	EXCLUSION
0.1	1.9	−	++	+++
0.2	1.8	±	++,+++	++++
0.3	1.7	+,++	+++	++++
0.4	1.6	+++	++++	++++
0.5	1.5	++++	++++	++++

For the next test, the sensitivity test, a mixture of 0.3 c.c. syphilitic and 1.7 c.c. negative serum were taken (Table II).

After establishing the appropriate dilutions of the sixteen tested antigens, the sensitivity test was undertaken with at least 30 sera from patients with various forms of syphilis and with other diseases as well as from normal

*Difco Laboratories, Inc., Detroit, Mich.

TABLE II. SENSITIVITY TEST

(TESTED SERUM.—KLINE TEST: DIAGNOSTIC +, ++; CONTROL, +++; EXCLUSION, ++++)

DILUTION OF ANTIGEN	E 1	E 2	E 3	E 4	1	2	3	4	AC 1	AC 2	AC 3	AC 4	PE 1	PE 2	PE 3	PE 4
1:100	−	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−
1:90	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	−
1:80	−	−	−	−	−	−	−	−	−	±	−	±	−	+++	−	−
1:70	−	±	−	−	−	−	−	−	±	+	−	±	−	+++	−	−
1:60	+	+	−	−	−	−	−	−	+	++	±	+	−	++++	−	−
1:50	++	+	−	−	−	−	−	−	++	+++	+	+++	−	++++	−	−
1:40	++	++	−	−	−	+	−	−	++	++++	++	++++	+	++++	±	+
1:30	+++	+++	−	+	−	++	−	−	+++	++++	++	++++	++	++++	+	++
1:20	+++	++++	−	++	−	++	+	−	++++	++++	+++	++++	++	++++	++	++
1:10	++++	++++	±	++	+	+++	++	+	++++	++++	+++	++++	+++	++++	+++	+++
1:5	++++	++++	+	+++	++	++++	++	+	++++	++++	++++	++++	++++	++++	+++	++++

TABLE III. SPECIFICITY TEST

DIAGNOSIS AND KLINE TEST*	E 1 1:60	E 2 1:65	E 3 1:85	E 4 1:30	1 1:10	2 1:40	3 1:25	4 1:15	AC 1 1:70	AC 2 1:80	AC 3 1:60	AC 4 1:80	PE 1 1:40	PE 2 1:105	PE 3 1:45	PE 4 1:55
Treated syphilis +, ++ +++ ++++	+	+	±	+	+	+	±	±	+	+	±	+	+	++	±	+
Normal − − −	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Normal − − −	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Active syphilis ++++ ++++ ++++	++	+++	++	+++	+	++	+	+	++++	++++	+++	++++	++	++++	++	++
Latent syphilis ± +, ++ +++, ++++	+	++	+	−	±	++	+	±	+++	++	−	−	±	+++	−	−
Cord blood − − −	−	+	−	++	++	+	+	−	+	−	−	−	−	−	−	−
Uterine blood − − −	++	±	+	++	++	+	−	−	−	−	−	−	+	−	−	−
Latent syphilis − ± +	+	±	+	+	+	±	−	++	++	++++	+	+++	++	++	+	+
Carcinoma of uterus − − −	−	+++	−	−	−	−	−	−	+	−	++	+	−	−	±	−
Pneumonia − − −	−	++	−	−	−	−	−	−	+	−	++	−	−	−	+	−

*Kline results are recorded here in three columns; they refer to diagnostic, control and exclusion tests, respectively.

TABLE IV. CHEMICAL COMPOSITION AND CLINICAL VALUES OF SIXTEEN ANTIGENS STUDIED

			20 GM. BEEF HEART POWDER								10 GM. BEEF HEART POWDER: FIRST EXTRACTION WITH ACETONE AND SECOND WITH PETROL ETHER				10 GM. BEEF HEART POWDER: FIRST EXTRACTION WITH PETROL ETHER AND SECOND WITH ACETONE			
			THREE TIMES EXTRACTED WITH ETHER; SOLUBLE PARTS ARE WEIGHTED				REMAINING ETHER EXTRACTED POWDER FIRST EXTRACTED WITH ACETONE AND THEN WITH PETROL ETHER											
			MARK	WEIGHT (GM.)	0.2 C.C. OF A DILUTION OF	RANK	MARK	WEIGHT (GM.)	0.2 C.C. OF A DILUTION OF	RANK	MARK	WEIGHT (GM.)	0.2 C.C. OF A DILUTION OF	RANK	MARK	WEIGHT (GM.)	0.2 C.C. OF A DILUTION OF	RANK
ACETONE SOLUBLE	PETROL ETHER	SOLUBLE	E 1	0.028	1:60	VI	1	0.050	1:10	XIV	Ac 1	0.160	1:70	IV	Pe 1	0.197	1:40	IX
		INSOLUBLE	E 2	0.596	1:65	V	2	0.405	1:40	VIII	Ac 2	0.860	1:80	II	Pe 2	0.440	1:105	I
ACETONE INSOLUBLE	PETROL ETHER	SOLUBLE	E 3	0.410	1:8.5	XVI	3	0.495	1:25	XIII	Ac 3	0.600	1:60	VII	Pe 3	0.520	1:45	XII
		INSOLUBLE	E 4	0.172	1:30	XI	4	0.040	1:15	XV	Ac 4	0.090	1:80	III	Pe 4	0.260	1:55	X

patients. Examples are given in Table III. The antigen emulsions were used in the dose of 0.2 c.c. of the evaluated dilution.

According to their efficiency as syphilitic antigens, the sixteen antigens may be divided into four groups:

1. The best antigens were Pe 2 and Ac 2, each acetone-soluble-petrol ether-soluble fractions prepared without previous ether extraction. Ac 4, which is acetone soluble but petrol ether insoluble, was nearly as efficient as Ac 2.

2. Of medium value were Ac 1, E 2, E 1, and Ac 3, the three first mentioned being acetone soluble. They all had a tendency to react positively with sera from patients with nonsyphilitic diseases.

3. The equally efficient antigens 2, Pe 1, and Pe 4 were not sensitive enough and were nonspecific.

4. The remaining six antigens were very weak and nonspecific. (See Table IV.)

The most important part in every serodiagnostic test for syphilis is the specificity. The more sensitive the tests became, the more they approached the dangerous zone of nonspecific reactions. As shown in a previous paper,[10c] every antigen has five zones depending upon the dilution: (1) only negative results with all sera, (2) syphilis positive, nonsyphilitic negative, (3) syphilis and other diseases positive, normal persons negative, (4) all sera positive, and (5) self-inhibition. Only the second zone is specific for syphilis.

From this viewpoint the sixteen antigens could be divided into four groups (the Roman figures indicate the position in the sensitivity table [see Table IV]):

A. Absolutely specific were Pe 2 (I), Ac 2 (II), Ac 4 (III), 4 (XV), Pe 4 (X).

B. Slightly nonspecific were E 3 (XVI), 3 (XIII), E 1 (VI), Pe 1 (IX), and Pe 3 (XII).

C. Medium positive nonspecific reactions were shown by E4 (XI), 1 (XIV), and 2 (VIII), with cord or uterine blood. Ac 3 (VII) reacted more with sera from patients with diseases such as pneumonia, cancer, leucemia, etc.

D. Nonspecific reactions with all types of nonsyphilitic sera were shown by Ac 1 (IV) and E 2 (V), the latter yielding the most and strongest of the nonspecific reactions.

The positive reactions with nonsyphilitic sera were usually stronger than the positive reactions with sera from syphilitic patients. It may be possible to achieve with these nonspecific antigens an inverse verification test. Further investigation on this interesting phenomenon is being carried out and a special paper on the subject will soon be published.

DISCUSSION

Since Wassermann, Neisser, and Bruck first published their test for syphilis, a steady development in the preparation of antigens has taken place. The watery emulsion of fine particles from syphilitic fetal livers was followed by an alcoholic extraction of the same organ. It was replaced by the whole alcoholic extraction of normal organs, usually heart muscle of beef or man. These antigens were made more sensitive by adding cholesterol. Noguchi and Bronfenbrenner[11] introduced the acetone-insoluble fraction of alcoholic extractions of beef heart as antigen. Similar antigens were prepared by Bordet and Ruelens,[12] Wadsworth,[13] Kolmer,[14] and others. Scaltritti[15] used an alcoholic

solution of cadmium chloride to precipitate a certain group of lipoids. Through absorption of tissue extracts on aluminum hydroxide and on rare earths, such as bentonite and floridin, Balbi[16] tried to obtain a more specific antigen.

From the chemical viewpoint, the most extensive investigations of "lecithin-like substances of the myocardium" were performed by Erlandsen[17] (1907). He recommended the abolition of the old conception of lecithin and replaced it with a new and more comprehensive one in which the phosphatides were divided into four groups:

1. Monoamido-monophosphatide; the relation between nitrogen and phosphorus is 1:1; it consists of lecithin and cephalin.

2. Monoamido-diphosphatide; the relation between nitrogen and phosphorus is 1:2; it consists of cuorin.

3. Diamido-monophosphatide; the relation between nitrogen and phosphorus is 2:1; it consists of amidomyelin and sphingomyelin.

4. Diamido-diphosphatide; the relation between nitrogen and phosphorus is 2:2; it does not exist in the myocardium.

Ten years later (1917) Neymann and Gager[18] published a new method for making Wassermann antigens from normal heart tissue which was partly based on Erlandsen's analysis of the different phosphatides. In one experiment they extracted dried pulverized beef heart with ether, acetone, and alcohol. In another experiment they used, as starting material, the secondary alcohol extraction and treated this with ether, acetone, and alcohol. They found "that lecithin is the only substance of the primary ether extract that has any antigenic value, and that the most important substance, as regards antigenic value, occurs in the secondary alcohol extract and is a diamino-monophosphatide."

Pangborn[19] isolated a new serologically active phosphatide from beef heart by a special method of extraction. It was named cardiolipin and is essential for the reactivity of beef heart antigens in the serologic tests for syphilis. It is important to note that it must be prepared from fresh beef heart tissue by a complicated method: precipitation by $BaCl_2$ from methyl alcohol extracts of tissue and successive purification over barium and cadmium salts.

Few of the mentioned phospholipides can be compared with the fractions prepared by the method described in this paper. The antigens are not chemically pure but are a mixture of different phospholipids. For example, E 1 and E 2 can be compared with the B of Neymann and Gager, fats with slight amount of phosphatides and a complement fixating power of 1:1600; E 3 can be compared slightly with the A of Neymann and Gager, which has no binding power. E 4 is perhaps a combination of C (lecithin), D (cephalin), and E (cuorin). Of these, only C has the higher binding power 1:800.

Another approach to eliminate the nonspecific lipoids was made by the introduction of spirochetal antigens. Spirochetal antigens were first prepared by Hinklemann,[20] Gaehtgens,[8] and Klopstock.[9] At the Washington Serology Conference, two serologists (Eagle and Kolmer), used spirochetal antigens, but the results were not as satisfying as expected from previous reports by other authors. In a previous publication,[10e] Klopstock's original spirochetal antigen was shown to be very good when it was used with heated sera.

Extraction with petrol ether was first recommended by Klosterman and Weisbach[21] because it extracts less of the active substances. They used it only as a preliminary extraction before making the real one and made no use of

the petrol ether-soluble lipoids. The comparison of the petrol-soluble and -insoluble lipoids shows that the soluble ones also can be used successfully for diagnosis of syphilis as shown in Tables I to IV. The slight preponderance of sensitivity of the antigen gained through a previous petrol ether extraction over the antigen gained through a previous acetone extraction means that the first-mentioned procedure gives more efficient lipoids in spite of the fact that the quantity is only about one-half (0.440 Gm.) of the amount gained by a previous acetone extraction (0.860 Gm.).

The main difference between both procedures lies in the greater amount of acetone-soluble lipoids yielded by a previous acetone extraction (1.020 Gm.) as against 0.637 Gm. with a previous petrol ether extraction. The amount of the acetone-insoluble fractions is almost identical. When the first extraction was made with acetone, 0.690 Gm. of the acetone-insoluble fractions was obtained; when petrol ether was used for the first extraction, 0.780 Gm. was gained.

CONCLUSION

1. The most effective fraction of the lipoids of dried beef heart is the petrol ether-soluble one.

2. The acetone-soluble fraction of petrol ether-soluble fraction is more sensitive than the acetone-insoluble fraction.

3. Whether the extraction is first made with acetone and then with petrol ether or reversed does not make too much difference, but the former procedure seems to yield a slightly superior antigen.

4. A previous ether extraction renders antigens of inferior value, the best of them (E 2 and E 1) occupying the fifth and sixth place in sensitivity and having very low specificity.

5. The petrol ether- and acetone-insoluble fractions (Pe 3 and Ac 3) can be used to verify a nonspecific positive reaction inasmuch as they give a stronger positive reaction with nonsyphilitic sera than do the "specific" antigens. Syphilitic sera either do not render a positive reaction with these antigens at all or one much weaker than the "specific" antigens.

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A STUDY OF THE RELATIVE SENSITIVITY OF DIFFERENT LOTS OF ANTIGEN EMPLOYED IN THE SEROLOGIC TESTS FOR SYPHILIS

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THE Kahn tests have been routinely employed in the laboratories of the Georgia Department of Public Health for many years. Antigens used have been either those prepared in our laboratory and corrected to standard requirements by Kahn's laboratory or those approved by Kahn and purchased from commercial firms.

Before a new lot of antigen was placed in use, it was run in parallel with the current lot of antigen on several series of weak-reacting sera. In this way, the relative sensitivity of the two antigens in routine work could be determined. Some antigens compared quite favorably, while others showed more marked differences in sensitivity.

If the level of sensitivity had gradually been lowered or elevated by successive antigens, then a new lot of antigen, with a reverse trend, would tend to restore the sensitivity approximately to the desired level. If the sensitivity of the test continued to be lowered or elevated beyond certain expected comparable limits by a new lot of antigen, it caused concern, but the antigen was nevertheless used, since it had been approved for a satisfactory sensitivity level by Kahn's laboratory.

From the Georgia Department of Public Health.
Received for publication, Sept. 22, 1945.

A Kahn antigen with an observed low sensitivity was placed in routine use and was employed in the 1944 National Serology Evaluation Study. A poor rating in sensitivity, compared to Kahn's laboratory and to other tests employed in our laboratory on these specimens, served as a stimulus for this study.

Arrangements were made with one of the local venereal disease clinics to furnish specimens of blood from patients receiving antisyphilitic treatment. Lists of names of patients who had given weak or moderate reactions by the current lot of antigen in routine use in our laboratory were furnished to the clinic at weekly intervals. The clinicians secured from these patients, upon their next visit to the clinic, ample specimens of blood and forwarded them to the laboratory.

Seven approved Kahn standard antigens were secured for this study. Sources of these antigens are listed at the conclusion of this paper.

The same technician performed all tests in parallel with all antigens. The specimens were examined in groups of five so as to conform to the time requirements in the use of the antigen emulsions. Precautions were taken to circumvent factors which might be responsible for differences in results.

Antigen suspensions were prepared and used in numerical sequence beginning with antigen 1 in the first group of specimens, antigen 2 in the second group, and so on in rotation throughout the study, so that no antigen would be given any favored position. A separate pipette was used to introduce each antigen emulsion into the test tubes.

The sera were introduced with a separate nondelivery pipette into all antigen emulsions in the same order that antigens were introduced. Each serum was allowed to stand with its antigen mixture for the same length of time, between three and ten minutes, before the three-minute shaking period. Controls on each antigen were run with each group of specimens.

The tubes for the tests were set up so that a separate rack contained the test on each serum with all the antigens. The first set of tubes in the first rack was diluted with saline and read immediately, and then the remaining sets in the numerical order of the antigens were treated in like manner. The other racks were diluted and read in sequence in the same way. A fifteen-minute reading was also made on all tests.

Some sera were found to give 3 or 4 plus reactions by all antigens. These results were excluded from the study. A quantitative test was performed on each of these sera and a dilution prepared which would apparently be critical for all antigens. The tests were repeated on these saline dilutions of sera and the results recorded.

The results on the 100 specimens examined by the Kahn standard test in this study are given in Table I, and the difference in sensitivity of the seven antigens is shown. The classification of the results from the tube readings is the same as that used for routine reporting and follows very closely the recommendation of Kahn. Sensitivity percentages are calculated on the same basis employed in the National Serology Evaluation Studies.

Dispersibility of the precipitates between the immediate and fifteen-minute readings was much more pronounced with some antigens than with others. It will be observed (Table I) that antigen 4, the most sensitive, showed the least dispersibility, a drop of 7.5 per cent in sensitivity, and that antigen 6, the next highest in sensitivity, exhibited the greatest dispersibility, a drop of 21.5 per

TABLE I. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE KAHN STANDARD TEST

ANTIGEN	1	2	3	4	5	6	7
			Immediate Reading				
Positive	61	62	68	93	55	82	59
Doubtful	23	25	20	5	22	14	22
Negative	16	13	12	2	23	4	19
Sensitivity	72.5	74.5	78.0	95.5	66.0	89.0	70.0
			Fifteen-Minute Reading				
Positive	39	47	51	81	25	53	36
Doubtful	32	35	33	14	43	29	30
Negative	29	18	16	5	32	18	34
Sensitivity	55.0	64.5	67.5	88.0	46.5	67.5	51.0
			Average of Above Readings				
Positive	47	52	63	83	41	70	45
Doubtful	29	31	23	15	31	19	27
Negative	24	17	14	2	28	11	28
Sensitivity	61.5	67.5	74.5	90.5	56.5	79.5	58.5

cent. Antigens 3 and 6 showed 11 per cent difference in sensitivity in the immediate readings and yet gave identical sensitivity in the fifteen-minute readings.

Taking the average of the immediate and fifteen-minute readings as a basis for comparison, antigen 4 showed the highest sensitivity (90.5 per cent) and antigen 5 the lowest (56.5 per cent), a spread of 34 per cent between these extremes.

It was then decided to repeat this study on another group of 100 specimens with a different group of five antigens selected from the sources listed at the conclusion of this paper. Specimens were secured in the same manner as for the first study and all of the same safeguards of technique which might influence the results were observed. In this study with five antigens, the specimens were examined in groups of seven. Specimens giving strong reactions were diluted with saline to the critical point for the antigens.

The comparison of the sensitivity of the antigens in the immediate, fifteen-minute, and average readings is given in Table II. It will be noted that the dispersibility of the precipitate in the two most sensitive antigens, 10 and 12, and the least sensitive antigen, 8, was approximately the same and was much less than for the other two antigens.

Based on the average readings, antigen 10 was the most sensitive (95.5 per cent) and antigen 8 the least sensitive (39.0 per cent), a difference of 56.5 per cent between these extremes. Antigens 10 and 12 were quite comparable in sensitivity in all readings.

It was then decided to determine if this observed variation in sensitivity was peculiar to the Kahn standard antigens, or if the antigens employed in the other recognized serologic tests would exhibit similar differences in sensitivity. Antigens were secured for this part of the study from sources listed at the conclusion of this paper. It will be noted, in this connection, that one antigen was secured from the laboratory of each author-serologist.

Specimens were secured in the same manner as for the comparison of the Kahn antigens. The techniques of the authors of the tests were rigidly observed. All possible safeguards were set up to insure parallel performance in every detail. Classification of plus reactions into positive, doubtful, or negative was followed as recommended by each author.

TABLE II. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE KAHN STANDARD TEST

ANTIGEN	8	9	10	11	12
		Immediate Reading			
Positive	28	67	96	75	92
Doubtful	43	27	4	15	5
Negative	29	6	0	10	3
Sensitivity	49.5	80.5	98.0	82.5	94.5
		Fifteen-Minute Reading			
Positive	19	35	77	33	72
Doubtful	34	43	18	37	20
Negative	47	22	5	30	8
Sensitivity	36.0	56.5	86.0	51.5	82.0
		Average of Above Readings			
Positive	20	52	93	61	87
Doubtful	38	34	5	26	11
Negative	42	14	2	13	2
Sensitivity	39.0	69.0	95.5	74.0	92.5

The different sensitivity levels of the various tests precluded the acceptance of the same group of specimens for all tests. While more than one test was performed on many of the specimens, they may be considered for the purpose of this study as a series of 100 different specimens for each test.

To illustrate, the Kline and Mazzini tests may have been performed on the same specimen. If the three antigens used in the Kline test gave very weak reactions and any one of three antigens used in the Mazzini test gave less than a 4 plus reaction, the specimen was accepted as critical for both tests. If any one of the antigens in the Kline test gave less than a 4 plus reaction and all three Mazzini antigens gave 4 plus reactions, the specimen was discarded from consideration in the Mazzini test series. If, on the other hand, the Mazzini antigens showed variation in results and all Kline antigens gave negative reactions, the specimen was not considered critical for the Kline test.

Specimens giving 4 plus reactions by all antigens in any given test were diluted serially and the test repeated in quantitative manner. The lowest dilution showing variation in plus reactions was accepted as the critical point for the antigens in that test.

It will be noted from Table III that Kline antigens 1 and 2 gave quite comparable results. Antigen 3 was far less sensitive.

The relative sensitivity of three antigens employed in the Eagle microflocculation test is shown in Table IV. Antigen 2 was found to be much less sensitive than either of the other two antigens.

TABLE III. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE KLINE DIAGNOSTIC TEST

ANTIGEN	1	2	3
Positive	62	61	39
Doubtful	34	33	28
Negative	4	6	33
Sensitivity	79.0	77.5	53.0

TABLE IV. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE EAGLE MICROFLOCCULATION TEST

ANTIGEN	1	2	3
Positive	67	26	55
Doubtful	28	21	34
Negative	5	53	11
Sensitivity	81.0	36.5	72.0

TABLE V. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE HINTON FLOCCULATION TEST

ANTIGEN	1	2	3
Positive	70	45	61
Doubtful	28	39	28
Negative	2	16	11
Sensitivity	84.0	64.5	75.0

TABLE VI. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE MAZZINI FLOCCULATION TEST

ANTIGEN	1	2	3
Positive	60	74	68
Doubtful	39	26	31
Negative	1	0	1
Sensitivity	79.5	87.0	83.5

It will be observed from Table V that there is a difference of 19.5 per cent in the sensitivity of antigen 1 and antigen 2 used in the Hinton test.

From Table VI it may be seen that the antigens used in the Mazzini test compared rather favorably in sensitivity.

In the Kolmer test, it was more difficult to secure specimens which would reflect marked differences in sensitivity based on positive, doubtful, and negative results as classified by Kolmer. For instance, a specimen giving 1 plus, 2 plus, and 4 plus reactions by three different antigens would be recorded as positive by all three antigens.

Therefore, in order to maintain a sensitivity level of the Kolmer test in a relative position with the other serologic tests, it was necessary to incorporate more of the type of specimens which gave very weak reactions.

It will be observed from Table VII that antigens 1 and 3 in the Kolmer test compared very favorably in sensitivity. Antigen 2 was found to be much less sensitive than the other two antigens.

TABLE VII. RESULTS OBTAINED ON 100 SPECIMENS OF BLOOD EXAMINED BY THE KOLMER SIMPLIFIED TEST

ANTIGEN	1	2	3
Positive	69	46	69
Doubtful	27	14	25
Negative	4	40	6
Sensitivity	82.5	53.0	81.5

COMMENTS

This study is admitted to be rather critical in that it includes an unusually large number of doubtful reactions which magnify differences in sensitivity. In routine work such differences are diluted with large numbers of definitely positive or negative reactions.

Nevertheless, serology evaluation studies incorporate a comparatively large number of weak-reacting specimens, and acceptable performance is determined by the results reported. This study seeks to show that failure to achieve satisfactory ratings in sensitivity may lie in the antigens employed and may not always reflect a lack of skill in performance.

As one lot of antigen is exhausted and another placed in use, laboratories should be able to maintain approximately the same level of sensitivity. Otherwise, specimens for recheck purpose, which give differences in results, may

lead to disconcerting situations. Particularly this may be reflected in quantitative tests.

From the rather wide variation observed in the sensitivity of some of the antigens employed in this study, it appears that more attention should be given to the standardization of this reagent. This could probably be better accomplished by the establishment of a special laboratory under some agency for the production, standardization, and distribution of all antigens employed in the various recognized serologic tests for syphilis.

SUMMARY AND CONCLUSIONS

Three or more different lots of approved antigens were run in parallel in each of the following serologic tests for syphilis: Kahn standard, Kline diagnostic, Eagle microflocculation, Hinton flocculation, Mazzini flocculation, and Kolmer simplified complement fixation. Antigens were secured from various sources, including at least one from the laboratory of each author-serologist. One hundred specimens of blood for each test were secured from syphilitic donors under treatment.

The technique of each author was rigidly followed. Throughout the study, all precautions for parallel conditions were observed for each procedure. Reactions were classified as positive, doubtful, and negative as recommended by each author for his test.

Specimens giving negative reactions by all antigens in any test were excluded from consideration. Specimens giving 4 plus reactions by all antigens in a given procedure were diluted serially and re-examined in a quantitative manner. The lowest dilution giving less than a 4 plus reaction by any of the antigens was considered as critical for the antigens of that test.

Sensitivity was calculated on the same basis as adopted in the National Serology Evaluation Studies. A rather wide variation was found in the sensitivity of the antigens in all the tests with the exception of the Mazzini test. This observation in the Mazzini test should be considered as negative information, since the number of antigens employed was too small to be conclusive in this respect.

This study was more critical for the sensitivity of antigens than would be experienced in routine work. However, differences in sensitivity ratings achieved in serology evaluation studies by different laboratories employing the same test may be due to the relative sensitivity of the antigens used.

It is felt that the quality of laboratory service would be further improved if a special laboratory could be established under some agency for the preparation, standardization, and distribution of all antigens employed in the serologic tests for syphilis.

The authors wish to acknowledge the excellent cooperation of Dr. E. S. Armstrong and Mrs. Mary E. Daniel, R.N., of the Atlanta Health Center, and Dr. W. A. Mason and Carrie Lou McCarter, R.N., of Westside Health Center, in securing the specimens for this study.

Kahn Antigens

1. University Hospital Laboratories, Ann Arbor, Mich., Lot No. 67 A.
2. Georgia Department of Public Health, Atlanta, Ga., Lot No. K-1.
3. Georgia Department of Public Health, Atlanta, Ga., Lot No. F.
4. Digestive Ferments Co., Detroit, Mich., Lot No. 45759.
5. Digestive Ferments Co., Detroit, Mich., Lot No. 46846.
6. Digestive Ferments Co., Detroit, Mich., Lot No. 47452.
7. Alabama Department of Public Health, Montgomery, Ala., Lot No. 29 A.

8. University Hospital Laboratories, Ann Arbor, Mich., Lot No. 71.
9. Digestive Ferments Co., Detroit, Mich., Lot No. 47452.
10. V. D. Research Laboratory, Staten Island, N. Y., Lot No. 8 (1944).
11. Navy Medical Center, Washington, D. C., Lot No. 89 B.
12. Army Medical Center, Washington, D. C., Lot No. 171.

Kline Antigens

1. LaMotte Chemical Products Co., Baltimore, Md., Lot No. 241.
2. LaMotte Chemical Products Co., Baltimore, Md., Lot No. 242.
3. Dr. B. S. Kline, Cleveland, Ohio (lot number not given).

Eagle Antigens

1. Digestive Ferments Co., Detroit, Mich., Lot No. 46151.
2. District of Columbia Department of Health, Washington, D. C. (lot number not given).
3. Dr. Harry Eagle, Baltimore, Md. (lot number not given).

Hinton Antigens

1. Digestive Ferments Co., Detroit, Mich., Lot No. 44163.
2. Maryland Department of Public Health, Baltimore, Md., Lot No. 18 X.
3. Dr. William A. Hinton, Boston, Mass., Lot No. 224.

Mazzini Antigens

1. Eli Lilly and Co., Indianapolis, Ind., Lot No. 3278-366276.
2. Eli Lilly and Co., Indianapolis, Ind., Lot No. 0169-350122.
3. Mr. L. Y. Mazzini, Indianapolis, Ind. (lot number not given).

Kolmer Antigens

1. Digestive Ferments Co., Detroit, Mich., Lot No. 46071.
2. Digestive Ferments Co., Detroit, Mich., Lot No. 43450.
3. Research Institute of Cutaneous Medicine, Philadelphia, Pa. (lot number not given).

THEORY OF THE COLLOIDAL GOLD REACTION

I. Reactions Between Gold Sol and Isolated Protein Fractions

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SCHULZ and Zsigmondy's gold number[1] was the incentive which led to the development of the diagnostic gold reaction in cerebrospinal fluid.[2] The gold number, the minimum amount of a protein (colloid) that protects 10 c.c. of formol gold against coagulation by 1 c.c. of 10 per cent saline, had been used only for characterizing isolated protein fractions and not for mixtures such as occur in cerebrospinal fluids. Application of the gold number to such specimens proved to be of minor significance.

In studying syphilitic cerebrospinal fluids, it was suspected that syphilis reagin, being an abnormal euglobulin fraction, was insoluble in the distilled water used as diluent in the gold number. Therefore, cerebrospinal fluids were serially diluted with 0.4 per cent saline before the formol gold was added. Various coagulation curves instead of the expected protection were obtained. The theoretical significance of the results was not realized at first. Interest was focused on the practical discovery that these curves seemed to be of clinical

From the Division of Laboratories and Research, New York State Department of Health.
Received for publication, Aug. 13, 1945.

TABLE I. REACTIONS WITH PROTEIN FRACTIONS AND THEIR MIXTURES

SERIAL NUMBER	MATERIAL		ALBUMIN-GLOBULIN RATIO	SERIAL DILUTIONS IN STRONG BUFFERS[3]														
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
				1:15	23	34	51	76	114	171	256	384	576	864	1296	1944	2916	4374
1	20 mg. per 100 c.c. albumin			0	1	1.5	2	1.5	1	0	0	0	0	0	0	0	0	0
2	20 mg. per 100 c.c. euglobulin			6.5	10	16	15	13	12	8.5	7.5	5	4	3.5	2.5	1.5	1	1
3	5 mg. per 100 c.c. pseudoglobulin			11	16	17	18	17	9	6.5	5.5	5	3.5	2	1.5	1	1	1
4	20 mg. per 100 c.c. pseudoglobulin			2.5	3.5	7	10	13	16	14	10	7.5	5.5	5	3	1.5	1	1
5	100 mg. per 100 c.c. pseudoglobulin			1	1	1	1.5	2	3	5.5	9.5	13	15	16	15	8.5	7.5	7
6	1,000 mg. per 100 c.c. pseudoglobulin			1	1	1	1	1	1	1	1	1	1.5	2	3	7	9.5	14
	PSEUDO-GLOBULIN IN MG. PER 100 C.C.	ALBUMIN IN MG. PER 100 C.C.																
7	1,000	Uniformly 100	0.1	1	1	1	1	1	1	1	1	1	1	2	3	6	10	15
8	300		0.33	1	1	1	1	1	1	1.5	1.5	2.5	4.5	7	13	16	15	13
9	100		1	1	1	1	1.5	1.5	2.5	4	6.5	9	10	13	10	9	7.5	6
10	30		3.3	1.5	1.5	2	3	5	7.5	10	13	11	9.5	7	5.5	4.5	4.5	3.5
11	10		10	1.5	2	3.5	5	6.5	8	7.5	6.5	6.5	5.5	4.5	4.5	3.5	3	2
12	5		20	1.5	2	3	4.5	6	5.5	5.5	5	4.5	4.5	2.5	2.5	2	2	1.5
13	2		50	1.5	1.5	2	3	2.5	2.5	2.5	2	2	1.5	1.5	1	1	1	1

The numerical values are obtained with the gold color standard and express the degree of coagulation of the gold sol. 9 corresponds to the unchanged red; 20 to colorless or complete coagulation; 10, to blue; 1-9, to red-blue; and 11-19, to blue-white.

value. Particularly, the so-called "paretic curve" quickly became indispensable in the prognosis of neurosyphilis.

A theoretical explanation of the gold reaction should clarify the origin and significance of these curves. Unfortunately, the results of the diagnostic test seem too complex for direct interpretation. Therefore, we have studied the various physicochemical factors conditioning the milieu and the action of known protein fractions and their mixtures (see Table I).

EXPERIMENTAL STUDY

Physicochemical Factors Conditioning the Reaction.—Experimental studies demanded a standardized technique controlling the physicochemical factors of the milieu. The "quantitative gold reaction"[3] supplied this. The most significant factor proved to be the pH.[4] Thus hemoglobin either coagulates or protects gold sol, depending on whether the pH is below or above 7.0. This experiment indicates that any statement about reactions between gold sol and protein is meaningless, unless the pH is fixed.

A second physicochemical factor was the comparatively rapid loss of sensitivity of citrate gold due to increasing dispersity. This has recently been eliminated in a simple way. In preparing citrate gold 1:10,000,[5] boiling was previously continued for three minutes after the red color appeared. By shortening the boiling time, higher turbidities are obtained, which are measured in a photoelectric colorimeter. The turbidity, as a rule, decreases by about 10 per cent during the first twenty-four hours at room temperature. By the new method, the boiling time is carefully regulated to obtain a turbidity of approximately 130. If the turbidity exceeds this value, the solution is stored until it comes down to this value. Then 0.3 c.c. of M/5 sodium hydroxide is added to each 100 c.c. of gold sol. The pH of citrate gold 1:10,000 is around 4.1; that of the neutralized gold sol reaches an equilibrium after several days around 7.1. The neutralized gold sols thereafter remain practically unchanged for from three weeks to two months. Presumably, the formol gold[2] previously used did not change in sensitivity as rapidly as citrate gold, because its pH was 7.4.

A third physicochemical factor, temperature, is of little influence. The only necessary precaution is that freezing and thawing be avoided, since they irreversibly coagulate gold sol.

A fourth physicochemical factor, the presence or absence of electrolytes, proved to be of cardinal significance. The presence of electrolytes is obligatory in the diagnostic test. The concentration must be kept constant within the optimal range. This range is wider than that of the pH, but the concentration selected must not be changed.

In order to conduct experiments in the virtual absence of electrolytes, two technical factors were introduced: (1) the use of pseudoglobulins, euglobulins being insoluble under the conditions of these experiments; and (2) the pH of the diluent and of the gold sols, which was fixed at 7.4. Distilled water was unsuitable, because its pH was below 6.0. Either distilled water neutralized with sodium hydroxide, under potentiometric control, to pH 7.4 or M/100 phosphate buffer of pH 7.4 was substituted. Formol gold has a pH of 7.4. Acid citrate gold was neutralized, under potentiometric control, to pH 7.4 and held until an equilibrium was reached. No separate neutralization of the citrate gold was required in the diagnostic test, because the strong buffers used as diluent furnished a uniform pH 7.4 throughout the dilutions.

Three Different Reactions of Proteins With Gold Sol.—When various protein fractions were mixed with gold sol, in the presence or absence of electro-

lytes, and pH 7.4 was maintained, three qualitatively different reactions were observed: protection, sensitization, and coagulation.

1. Albumins prepared from horse serum with ammonium sulfate, serially diluted with neutralized distilled water, exhibited either peptization or protection. Peptization, the opposite to coagulation, may be recognizable by a color change or decrease of turbidity. Protection was demonstrable by adding 10 per cent saline several minutes after mixture. In the presence of electrolytes, the results were approximately the same, except for a narrow zone of barely perceptible coagulation (see Table I, No. 1), probably due to contamination with globulin, since it decreased noticeably with repeated salting out or when the fraction precipitated above 60 per cent saturation of ammonium sulfate was used. Complete purification was not achieved with salting-out methods. Crystallized egg albumin produced the same zone.

2. Pseudoglobulin, after dialysis, was diluted with neutralized distilled water, and neutralized citrate gold was added. No coagulation occurred, but protection was demonstrated by the results of the gold number. When pseudoglobulin was diluted with strong buffers by the routine technique of the quantitative gold reaction, a coagulation curve was obtained (Table I, No. 3) characterized by a sharp maximum corresponding to a constant optimal ratio. An excess in the prezone elicited decreasing strength of coagulation and increasing protection or peptization. From these results, the current view expressed by Presser and Weintraub,[6] that "globulins coagulate gold sol," seems to be incorrect in two respects. Globulins protect in the prezone and, in the range of optimal ratio, sensitize gold sol. This phenomenon corresponds to the first phase of agglutination,[7] where a complex of bacteria and agglutinin is formed. In the second phase, electrolytes coagulate this complex just as they do the gold sol.

3. *True coagulators,* in contradistinction to sensitizers, are substances that coagulate gold sol in the virtual absence of electrolytes; that is, in a milieu so poor in electrolytes that pseudoglobulin elicits invisible sensitization. Deuteroalbumose is a representative coagulator. It is easily prepared from proteose peptone,* being the fraction precipitated between 50 and 100 per cent saturation with ammonium sulfate. A second representative occurs in paretic cerebrospinal fluids.[8] Since this pseudoglobulin-like substance implies parenchymatous degeneration, it might be called *degenerative protein.* Gamma globulin also proved to be a true coagulator; its relation to the similar substance in paretic cerebrospinal fluids is under investigation.

True coagulators are different from the preceding two groups in that they coagulate in the absence of electrolytes, exhibit no or a rapidly disappearing optimal ratio or prezone, and in the presence of electrolytes elicit far stronger coagulation over a wide range of the usual serial dilutions than normal globulins. This difference explains why degenerative protein invariably elicits the most characteristic plateau curve.

Gold Reaction and Albumin-Globulin Ratio.—In the original description of the gold reaction,[2] the assumption was ventured that the albumin-globulin ratio played a role in producing the various types of curves. A modification of this hypothesis has been widely adopted. The theory was based on the assumption that globulins coagulate and albumins protect gold sol and that the different types of responses are dependent on various mixtures. Subsequent experiments have discredited this explanation. The albumin-globulin ratio appears to be of little significance.

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Previous experiments[8] proved the paretic curve to be independent of a definite albumin-globulin ratio. The question remained as to how far the ratio might be responsible for other types of curves, the prezone curves. The hypothesis that the albumin-globulin ratio is of decisive significance to the gold reaction of course implies that different globulin fractions react identically with gold sol.

In Table I, No. 2 and No. 4, are shown that identical concentrations of euglobulin and pseudoglobulin prepared from horse serum with ammonium sulfate yield characteristically different prezone curves. These simple experiments disprove the hypothesis that the albumin-globulin ratio is the only factor influencing gold reactions but fail to show what lesser role it may play. In testing various preparations of pseudoglobulin, it was learned that about 0.12 mg. per 100 c.c. gave maximal coagulation. The serial dilution of euglobulin was invariably higher, about 0.39 mg. per 100 c.c. In view of the incomplete purification obtained with salting-out procedures, it is not claimed that these numerical values are definitely established but only that euglobulin and pseudoglobulin give different quantitative reactions with gold sol.

If the albumin-globulin ratio is not the decisive factor, what does determine the location of the maximum in prezone curves? The problem has been investigated by testing isolated globulin fractions of known concentration in the presence and absence of albumins.

When pseudoglobulin solutions of increasing concentration have been tested (Table I, Nos. 3 to 6) as if they were cerebrospinal fluids of unknown constitution, the maximum has shifted increasingly to the right. In these quantitative experiments, the apparent shift to the right in the absence of albumins appears to be an expression of a *constant optimal ratio,* the maximum obtaining in those dilutions containing 0.12 mg. per 100 c.c. In other words, by testing globulin solutions of known constitution and concentration, the location of the maximum can be predicted. Inversely, when cerebrospinal fluids of unknown globulin content were tested, the maximum indicated the absolute "globulin" concentration. Although euglobulin and pseudoglobulin react differently, in mixtures such as occur in cerebrospinal fluids, their partial curves are superimposed so that the combination curve indicates only the effect of globulins as a group. The specificity of globulin fractions cannot be directly determined in the routine test.

The effect of albumins, or of the albumin-globulin ratio, upon the location of the maximum may be studied in two ways: by testing artificial mixtures of globulins and albumins and by testing cerebrospinal fluid before and after the removal of albumins.

Only negative results were obtained with artificial mixtures of euglobulins, pseudoglobulins, and albumins in attempting to produce a true paretic curve.[4, 8] A plateau curve resulted only in the presence of a *true coagulator.* The claimed parallelism between increasing albumin-globulin ratio and shift to the right[6] was based on experiments requiring albumins completely free of globulins, a precaution technically difficult to observe. We have measured the effect of an increasing albumin-globulin ratio by testing a globulin solution of known concentration, which located the maximum in one of the serial dilutions, and then adding increasing amounts of albumin. It was observed that the maximum shifted to the right.

In repeating these experiments, it was noted that the shift to the right produced by the admixture of albumins was widely different when various albumin preparations were used. Reznikoff[9] described the effect of albumin upon gold sol as producing complete coagulation over a wide range. Other authors have found the effect to be exclusively or predominantly protective.[5]

As has been mentioned, albumins exhibit decreasing coagulation when progressively purified by salting-out methods. This suggests that albumins which coagulate strongly have probably been contaminated with globulins. The same explanation was found to apply in our initial experiments, which inadvertently produced increasing concentration of globulins and thus caused a shift.

The use of protein fractions purified by electrophoresis might have been of interest. However, because all the previous experiments were performed with fractions separated with the conventional ammonium sulfate concentrations, the same procedure had to be employed. It was found that the use of albumin (horse serum), after repeated reprecipitation by 60 per cent saturation with ammonium sulfate, supplied definite results if the globulin rather than the albumin was varied. In Table I, Nos. 7 to 13, are shown the results of such experiments. If these results are interpreted by previous standards, the explanation would be that the maximum shifts to the left rather than to the right with increasing albumin-globulin ratio. Actually, these experiments prove that the changing location of the maximum has nothing to do with the albumin-globulin ratio. The maximum is exclusively determined by the absolute concentration of the globulins. The optimal ratio of 0.12 mg. per 100 c.c. remains constant in the presence or absence of albumins. Deviations from this constant optimal ratio appear only where comparatively low globulin concentrations are used (Table I, Nos. 11 to 13). The contamination of the albumins has a marked effect on the actual globulin concentration.

The real effect of albumin in albumin-globulin mixtures seems clearly demonstrated by these experiments, for in comparing identical globulin concentrations in the presence and absence of albumins, it appears that the maximum remains the same; only the strength of coagulation in the maximum is reduced as the albumin admixture increases.

These results suggest that the coagulation curves observed in the gold reaction are affected by the presence of albumins, or the albumin-globulin ratio, only to the degree that the strength of coagulation may be reduced by protecting albumins. This explanation of the relatively subordinate role of the albumin-globulin ratio applies also to the results obtained in cerebrospinal fluids as demonstrated by the following experience. Cerebrospinal fluids of high protein concentration were used. One specimen was from a patient with paresis and another from one with tuberculous meningitis; in the latter the protein concentration was approximately 150 mg. per 100 c.c. The precipitate obtained with 50 per cent saturation of ammonium sulfate was separated by centrifuging in an angle centrifuge and dialyzed against phosphate buffer of pH 7.4 used in the routine technique and diluted to the original volume. The only difference in gold curve due to the removal of the albumin was an increase in the strength of the reaction, the plateau of the paretic curve being slightly longer. The prezone curve obtained with the tuberculous cerebrospinal fluid was qualitatively the same, and only the strength of coagulation in the maximum was increased. In short, the results of the experiments with artificial mixtures seemed to be applicable to those obtained with cerebrospinal fluids, both in the paretic and prezone curves, indicating that the qualitative differences in the gold curves was unrelated to the albumin-globulin ratio.

SUMMARY AND CONCLUSIONS

The colloidal gold reaction has been studied by analyzing the physicochemical factors of the milieu and by testing various combinations of protein fractions.

Three qualitatively different reactions with gold sol have been demonstrated: (1) protection is produced both by albumins and normal serum globulins, in the prezone, and by hemoglobin at a pH above its isoelectric point; (2) sensitization is produced by serum globulin within an optimal range, electrolytes being required to complete the coagulation; (3) true coagulation, without the assistance of electrolytes, is produced in cerebrospinal fluid only by the pseudoglobulin-like *degenerative protein* indicative of parenchymatous degeneration which elicits the so-called paretic or plateau curve.

The plateau curve is fundamentally different from the remaining types, the prezone curves. The prezone curves in the absence of a *true coagulator* result from the qualitative and quantitative differences of the globulins, while the albumins, as protectors, have only a quantitatively decreasing effect. Euglobulin and pseudoglobulin react differently with gold sol; they may be characterized by the difference of their constant optimal ratio. This constant optimal ratio is the reason why, in prezone curves, the maximum shifts to the right with increasing globulin concentration.

Our interpretation ascribes to the albumin-globulin ratio the least important role in producing the qualitative differences of the various types. Since, according to a widely adopted hypothesis, the albumin-globulin ratio is the only factor producing these differences, the experimental data that are at variance with this assumption may be briefly summarized:

1. No mixture of globulins and albumins, no variation of the albumin-globulin ratio, is able to produce a plateau curve in the absence of a *true coagulator*.

2. Euglobulin and pseudoglobulin exhibit characteristically different optimal ratios.

3. The maximum does not shift to the right with increasing albumin-globulin ratio, as inferred from experiments in which albumins contaminated with globulins were used. The location of the maximum is entirely independent of the albumin-globulin ratio; it is regulated by qualitative and quantitative differences of the globulins.

4. The removal of albumins from cerebrospinal fluids, so that any effect of the albumin-globulin ratio is eliminated, produces no qualitative change either in the paretic curve or in the prezone curves.

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EXPERIMENTAL PRODUCTION OF PULMONARY EMBOLISM BY THE USE OF A VENOUS CATHETER

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MANY methods have been devised for the experimental production of pulmonary embolism. These methods readily fall into two groups, embolism and ligation. The method most commonly employed has been the introduction of various kinds of foreign bodies into the peripheral venous system. These have consisted of such miscellaneous substances as suspensions of barium sulfate, Prussian blue, and powdered charcoal, as well as larger particles such as blood clots, masses of fibrin, beads of enamel, lead shot, rubber finger cots, mercury, waxes, and many others. The choice of the size of the particles of the embolic material used has depended upon the size of the pulmonary vessels which the investigator has wished to occlude. Less frequently, various types of ligatures and clamps have been used to occlude partially or completely the lumen of the main pulmonary artery or its branches. Haggart and Walker[1] devised an ingenious method of clamping the pulmonary artery which enabled them to make a quantitative estimation of the degree of obstruction. The literature of experimental embolism has been reviewed by Bardin[2] (1934) and by Currens and Barnes[3] (1943).

Both methods have distinct and obvious limitations. The site of lodgment of the embolus cannot be adequately controlled once the mass has been introduced into the systemic vein, and its ultimate action and effect on the lung cannot be predicted with precision. Direct ligation has the disadvantage that the thorax must be opened with its consequent disturbances of pulmonary and cardiac dynamics, and usually the animal must be sacrificed immediately following the experiment.

The present communication is a description of a technique for the production of pulmonary embolism which appears to offer many advantages over hitherto published methods.

METHOD

Dogs were anesthetized by injecting nembutal (40 mg. per kilogram) intraperitoneally. The neck was shaved and one of the external jugular veins was exposed. Venous catheterization was performed by the method of Forssman[4] and Cournand and Ranges.[5] The animal was placed on its back on a fluoroscope table, and a No. 8 or No. 12 catheter, 60 cm. long with the tip bent at an angle of about 60 degrees, was inserted into the jugular vein. A slow drip of normal saline was maintained through the catheter to prevent clotting. Under direct fluoroscopic examination, the catheter was directed down the jugular vein through the superior vena cava and into the right auricle. The tip of the catheter was then turned posteriorly and to the animal's left and

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This investigation was aided in part by a grant from the John and Mary R. Markle Foundation.

Received for publication, Sept. 24, 1945.

directed through the tricuspid valve into the right ventricle. Further manipulation of the catheter with the curved tip in an upward direction resulted in the catheter being deflected from the opposite wall of the ventricle upward through the pulmonary valve and into the pulmonary artery. Once in the pulmonary artery it was possible to direct it into the right or left branch and into the smaller ramifications if so desired. Its position in a pulmonary artery may be ascertained with certainty by observing the tip of the catheter to lie outside the cardiac shadow when viewed in an anteroposterior or lateral position (see Fig. 1).

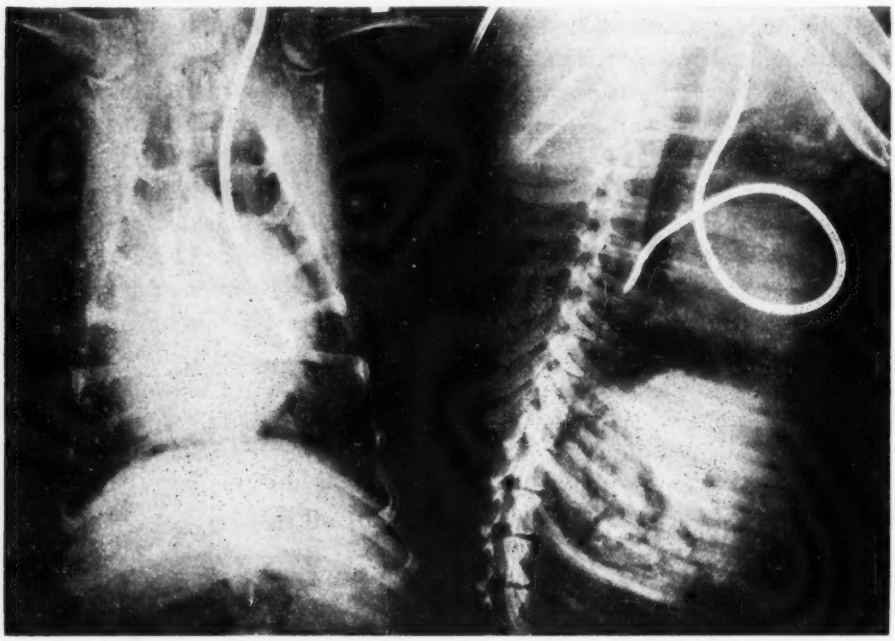

Fig. 1.—X-ray of a dog with catheter introduced through the jugular vein, superior vena cava, right auricle, right ventricle, and pulmonary artery. The tip of the catheter lies in a branch of the right pulmonary artery. Left, anteroposterior view: right, right lateral view.

With the catheter in the desired location, emboli may be injected directly into the pulmonary circulation. In this study, Japan wax has served as a useful medium for studying the location of the embolus in the lung because its melting point is low (40° C.) and it readily congeals at body temperature of the dog. Other embolic agents may be used with equal facility. An embolic substance with a high melting point cannot be used effectively due to the marked heat loss as the substance passes through the catheter with consequent plugging of the catheter. If such a substance is heated to a point which will offset the plugging of the catheter, it will be so hot when it reaches the lung that tissue destruction may occur. The addition of methylene blue or some other vivid coloring agent to the embolic material aids in its identification in the lung.

Because the thermal death point of tissue is approximately 47° C., care must be taken that any embolic material reaching the pulmonary circulation is below this temperature. In order to determine the degree of heat loss of the wax as it passed through the catheter, the following procedure was carried out. The catheters were immersed in a constant temperature water bath set at 40° C. which was continuously agitated. It was found that when the wax was heated to 45° C. and injected rapidly through a No. 12 catheter, the temperature of

the wax coming from the tip of the catheter was from 43 to 44° C. When the wax was heated to 85° C. and injected rapidly through a No. 8 catheter under similar conditions, the temperature of the effluent was from 45 to 46° C. It is assumed, therefore, that the temperature of the wax as it entered the pulmonary circulation was at approximately the same temperature.

The extent of the pulmonary vascular bed deprived of its blood supply can be readily controlled by the amount and type of embolic material used. In dogs weighing from 10 to 20 kilograms, 10 c.c. of Japan wax were sufficient to fill a lobular artery and cause the animal to die in from five to fifteen minutes. One animal, receiving only 3 c.c. of wax in a branch of the pulmonary artery supplying the right lower lobe, was still alive one hour later and was killed by pithing.

An example of the type of embolism obtained by the use of Japan wax is shown in Fig. 2. It should be noted that the embolus is in the form of a cast of the pulmonary artery and is quite reminiscent of emboli seen in massive pulmonary embolism in man.

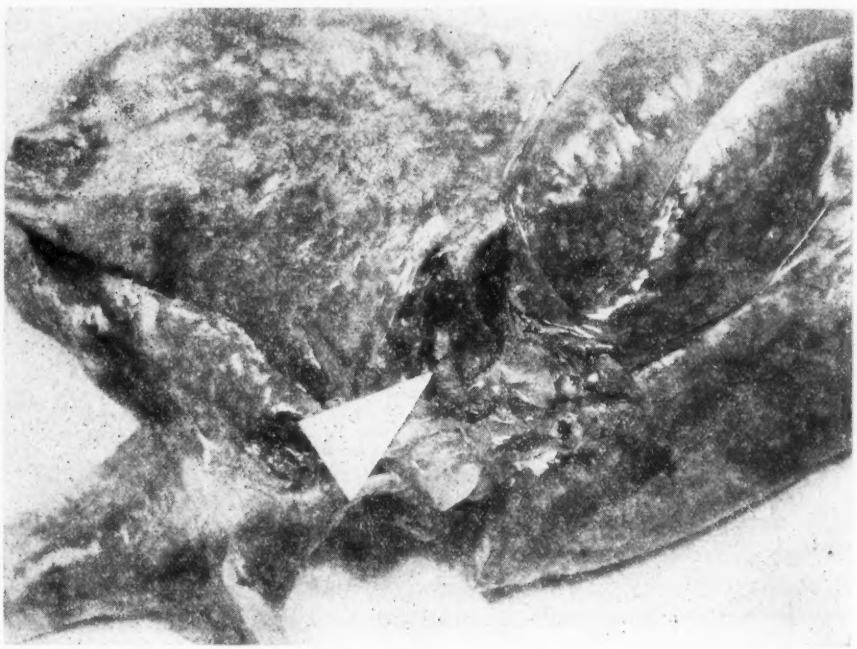

Fig. 2.—The arrow points to the wax cast of the pulmonary artery. Note the close resemblance to emboli found in pulmonary arteries in the human being.

The endothelium lining the jugular vein at the site of catheterization and that lining the vena cava, right auricle, right ventricle, and pulmonary arteries was carefully examined in eight dogs to determine if the catheter had traumatized the endothelium. The valves and papillary muscles were also examined. In no instance was there any evidence of damage to any of these structures.

DISCUSSION

We believe that the present method has definite advantages over those hitherto described. An embolus of any desired character may be introduced into any part of the pulmonary circulation at a given instant. By this technique it is not necessary to open the chest, thereby avoiding complicating disturbances of respiratory and circulatory dynamics.

Studies of the sequence of the pathologic physiology of pulmonary embolism are being carried out by the use of this general method.

SUMMARY

1. A method is described by which pulmonary embolism may be produced in animals in a controllable and localized area of the lung.

2. The method consists of introducing a catheter through the jugular vein and chambers of the right side of the heart into the main pulmonary artery or one of its smaller branches. The embolic substance is injected through the catheter.

3. It is felt that by the use of this method pulmonary emboli closely simulating those found in human beings can be reproduced.

The authors are indebted to Dr. Eugene M. Landis, for the use of the fluoroscope for this study, and to Dr. Roy E. Seibel, for roentgenologic assistance.

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PENICILLIN COMBINED WITH FEVER THERAPY

A Preliminary Report of Twenty Cases of Early Syphilis

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THE available literature offers few reports concerning penicillin-fever therapy. Barksdale[1] stated that penicillin combined with fever therapy was being satisfactorily used in cases of neurosyphilis. Struble and Bellows[2] reported on the favorable result obtained by the application of penicillin-fever therapy in a case of iridocyclitis of gonorrheal origin which had not responded to penicillin alone. Riba and co-workers[3] applied the cabinet type of therapeutic fever and penicillin to gonorrhea complications which had not responded to penicillin alone with encouraging results.

These data were not considered sufficient evidence that larger amounts of penicillin could be safely combined with physically induced fever or that penicillin activity could be demonstrated in the blood before, during, and after this type of therapeutic fever.

Physically induced fever combined with arseno-bismuth preparations has been employed by us in the intensive treatment of early syphilis[4, 5] and satisfac-

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Received for publication, June 16, 1945.

tory clinical and serologic results have been obtained. It was observed that by increasing the amount of these chemotherapeutic agents, fewer treatment failures occurred. Toxic reactions, however, were in direct proportion to the amounts of arsenical and/or bismuth[6] used in combination with therapeutic fever at the 106.0° F. (rectal) level.

It was natural, therefore, to combine penicillin with this form of fever therapy, substituting a chemotherapeutic agent whose toxicity has been negligible[7, 8, 9] as compared to arseno-bismuth preparations which have certain limitations when administered alone in large doses or in combination with therapeutic fever.

Twenty patients having primary or secondary syphilis (dark-field positive) were given penicillin-fever therapy. The amount of fever for each patient was three hours at 106.0° F. (rectal) level, produced in an air-conditioned cabinet (hypertherm). The sodium salt of penicillin was administered in the prefever period and during the course of the therapeutic fever. The patients all received a thorough diagnostic medical survey and the routine prefever preparation and postfever care.[10]

The first five patients received 25,000 units of penicillin intramuscularly by syringe every two hours during the twenty-four hours of the prefever period, a total of 300,000 units. The last dose was administered at 6:00 A.M. and the patients were in the fever cabinets at 8:15 A.M., reaching the 106.0° F. (rectal) level at approximately 9:15 A.M. Penicillin was administered intravenously by syringe in 100,000 unit doses at this time and again at the beginning of the second and third hours of the maintained fever. The amount of penicillin administered during fever therapy was 300,000 units, and the total amount for each patient was 600,000 units.

A second group of five patients was given the same routine treatment except that the penicillin was administered intramuscularly by syringe in 50,000 unit doses every four hours in the twenty-four hour prefever period.

Three of these ten patients had short periods of temperature elevation within the range of from 100.4 to 104.2° F. during the prefever penicillin administration. They were not ill and this was considered to be the Herxheimer reaction. During fever therapy nine of the patients showed no reactions. One patient (No. 6848) who had previously denied any drug addiction suddenly demanded a narcotic and became uncooperative when this was refused (he later admitted addiction); his treatment was discontinued for this reason after one and three-quarter hours of therapeutic fever. During the three postfever days all of the patients were ambulatory and in good condition.

In view of these favorable observations, it was decided to increase the total amount of penicillin to 1.2 million units for each of ten additional patients, 600,000 units to be administered in the twenty-four hour prefever period and 600,000 during fever therapy.

The patients were given the same routine fever therapy as was previously described. Penicillin was administered intramuscularly by syringe in 50,000 unit dosages every two hours in the twenty-four hours of the prefever period and 200,000 units intravenously by syringe at the height of fever and at the beginning of the second and third hours of maintained fever.

During the prefever penicillin administration one patient had a short period of temperature elevation to 100.2 and another to 101.8° F. These patients were not ill and this was interpreted to be the Herxheimer reaction. There were no reactions during fever therapy and the patients were alert and ambulatory during the postfever days.

In comparing the twenty patients treated with penicillin-fever therapy to patients receiving arsenicals, bismuth, and fever therapy, it was observed that during the fever, minor reactions were not evident and the patients' water balance was much less disturbed, and during the postfever period there was an absence of lethargy and fatigue with the penicillin-fever combination.

Blood penicillin determinations were made on twelve patients by the method described by Heilman and Herrell.[11] Blood specimens* were obtained from the antecubital vein after twelve and twenty-four hours of prefever penicillin administration, at the beginning of the maintenance period, and after one and two hours of maintained fever immediately before the intravenous penicillin was administered. Specimens were also obtained at approximately 8:00 A.M. on the first two consecutive postfever days. It is shown in Table I that penicillin activity in the blood is present during fever therapy and that there is marked variation in the values secured.

The clinical results obtained by these therapeutic methods have been of interest to us and have formed the foundation for further study of the combination of fever and penicillin in the treatment of early syphilis. The immediate clinical response to these three schedules of treatment was the same as evidenced

TABLE I. PENICILLIN LEVELS (O.U. OF ANTIBACTERIAL ACTIVITY PER CUBIC CENTIMETER OF BLOOD)

	PREFEVER			FEVER				POSTFEVER	
PATIENT	INTRAMUSCULAR SODIUM PENICILLIN ADMINISTRATION	12 HR. AFTER INITIATION OF PENICILLIN	24 HR. AFTER INITIATION OF PENICILLIN	INTRAVENOUS SODIUM PENICILLIN ADMINISTRATION	BEGINNING OF MAINTENANCE PERIOD	AFTER ONE HOUR	AFTER TWO HOURS	8:00 A.M. FIRST DAY POSTFEVER	8:00 A.M. SECOND DAY POSTFEVER
5212	25,000 O.U. q. 2 h. in 24 hr. prefever	0.48	0.06	100,000 O.U. after each blood specimen	None	0.24	0.48	None	None
6643	25,000 O.U. q. 2 h. in 24 hr. prefever	0.24	0.06	100,000 O.U. after each blood specimen	0.06	15.36	0.48	None	None
6654	25,000 O.U. q. 2 h. in 24 hr. prefever	0.48	0.06	100,000 O.U. after each blood specimen	0.06	30.00	15.36	None	None
6787	25,000 O.U. q. 2 h. in 24 hr. prefever	0.06	0.06	100,000 O.U. after each blood specimen	None	0.24	0.48	None	None
6801	25,000 O.U. q. 2 h. in 24 hr. prefever	0.48	0.03	100,000 O.U. after each blood specimen	None	0.24	0.48	None	None
6826	50,000 O.U. q. 4 h. in 24 hr. prefever	0.03	0.03	100,000 O.U. after each blood specimen	None	0.96	0.96	None	None
6830	50,000 O.U. q. 4 h. in 24 hr. prefever	0.24	0.03	100,000 O.U. after each blood specimen	None	0.48	0.96	None	None
6839	50,000 O.U. q. 4 h. in 24 hr. prefever	0.24	0.06	100,000 O.U. after each blood specimen	None	0.12	0.24	None	None
6848	50,000 O.U. q. 4 h. in 24 hr. prefever	0.48	0.03	100,000 O.U. after each blood specimen	None	15.36	0.48	None	None
6995	50,000 O.U. q. 4 h. in 24 hr. prefever	None	0.12	100,000 O.U. after each blood specimen	Trace	0.96	1.92	None	None
7523	50,000 O.U. q. 2 h. in 24 hr. prefever	0.24	0.24	200,000 O.U. after each blood specimen	0.03	0.96	1.92	None	None
7946	50,000 O.U. q. 2 h. in 24 hr. prefever	0.48	0.12	200,000 O.U. after each blood specimen	None	0.48	0.48	None	None

*The blood penicillin specimens were sent by air mail to the Mayo Clinic and the determinations performed under the direction of Dr. Paul O'Leary, Section on Dermatology, and Dr. D. H. Heilman, Division of Clinical Pathology, to whom we are indebted.

by any form of intensive treatment of syphilis. The lesions became negative for the *Treponema pallidum* on dark-field microscopy and healed. This will occur after penicillin in amounts of 60,000 units[12] is administered and should be expected after the amounts used in this study.

There are no reports to our knowledge on the treatment of early syphilis using 600,000 or 1.2 million units of penicillin within a period of forty-eight hours. Such amounts of penicillin have usually been administered over a period of seven and one-half days. The immediate clinical results with such schedules have been the same as experienced in this group of patients. This fact allows us only one method of comparison and that is the relapse rate.

The indicated eventual relapse rate using 600,000 units of penicillin over seven and one-half days is 40 per cent.[13] The ten patients treated with 600,000 units of penicillin in two days and three hours of fever at 106° F. (rectal) exhibited a relapse rate of 80 per cent. Although the group is small, the failure of eight of ten patients is certainly significant; all the failures occurred within the first six months after treatment, further proof of the inadequacy of this treatment schedule. The results in this group are shown in Table II.

TABLE II. NUMBER OF PATIENTS WITH SYPHILIS TREATED WITH FEVER AND 600,000 UNITS OF PENICILLIN FROM JULY 26 THROUGH AUG. 24, 1944, GROUPED BY FINAL DIAGNOSIS AND LAST REPORTED STATUS PRIOR TO JULY 1, 1945

		DIAGNOSIS	
		PRIMARY	
LAST REPORTED STATUS	TOTAL	SEROPOSITIVE	SECONDARY
Total	10	2	8
Negative	1	1	–
Positive—1 to 4 units	–	–	–
Positive—Over 4 units	1	–	1
Failures	8	1	7
Failures			
Total		8	
Clinical relapse	5		
Clinical progression	1		
Serologic relapse	2		

The indicated eventual relapse rate using 1,200,000 units of penicillin in early syphilis is from 15 to 20 per cent.[13] Two of the ten patients in this study treated by the use of 1,200,000 units of penicillin and three hours of fever at 106° F. (rectal) were not seen again after dismissal from the hospital. Two of the eight patients under observation have been classified as serologic relapses. No clinical failures have appeared in this group of patients. Both serologic failures occurred in the eighth month after treatment, at least two months later than any of the failures with 600,000 units. The clinical results in the group to date are encouraging and further study of the use of 1,200,000 units of penicillin plus physically induced fever are in progress. The serologic status to date of the patients in this group is shown in Table III.

TABLE III. NUMBER OF PATIENTS WITH SYPHILIS TREATED WITH FEVER AND 1.2 MILLION UNITS OF PENICILLIN FROM OCT. 2 THROUGH NOV. 8, 1944, GROUPED BY FINAL DIAGNOSIS AND LAST REPORTED STATUS PRIOR TO JULY 1, 1945

		DIAGNOSIS	
		PRIMARY	
LAST REPORTED STATUS	TOTAL	SERONEGATIVE	SECONDARY
Total treated	10	2	8
Not reporting	2	–	2
Total under observation	8	2	6
Negative	3	2	1
Positive—1 to 4 units	1	–	1
Positive—Over 4 units	2	–	2
Failures (serologic relapse)	2	–	2

SUMMARY

1. Preliminary evidence is presented to demonstrate that penicillin and physically induced fever may be safely combined in the treatment of early syphilis as described.

2. Penicillin-fever therapy as compared to arseno-bismuth-fever therapy was more comfortable for the patients.

3. Blood penicillin activity during penicillin-fever therapy was variable before and during this form of therapy, while none was detected on the two consecutive mornings after fever therapy.

4. The use of 600,000 units of penicillin plus three hours of fever at 106° F. (rectal) is inadequate treatment for early syphilis.

5. The use of 1,200,000 units of penicillin plus three hours of fever at 106° F. (rectal) is encouraging enough to warrant further study of the use of penicillin-fever therapy in early syphilis.

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PENICILLIN THERAPY IN HUMAN BARTONELLOSIS (CARRION'S DISEASE)

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THE therapeutic effectiveness of penicillin has not been established for human bartonellosis (Carrion's disease), an illness endemic in certain regions of South America, especially in Peru and Colombia. This paper describes the clinical course of two patients with bartonellosis who were treated with penicillin and records the changes which were observed in the morphology of the responsible organism and in the degree of erythrocyte parasitism.

The general aspects of Carrion's disease have been recently summarized by Rebagliati[1] and Weinman.[2] Briefly, this disease characteristically consists of two phases or stages. The first one, to which the names of Oroya fever, noneruptive, or hematic period are often applied, consists of a febrile period accompanied by an anemia which is usually severe, by signs of rapid red cell destruction, and by evidences of erythropoietic hyperactivity. The characteristics of this hemolytic anemia have also recently been summarized.[3] If the patient survives, he develops, after a variable period of time, an eruption of papular and nodular lesions which may develop in the cutaneous zone or in an internal organ. This second stage is sometimes referred to as verruga peruana, histioid phase or eruptive period. Frequent clinical variations are observed in regard to the clinical aspects and severity of these two phases. The mortality is high in the first stage of this disease and has been estimated to vary between 18 and 95 per cent, with a general average of about 40 per cent;[2, 4] it is thought that secondary infections are in part responsible for the high degree of mortality. In the second phase of the illness, the mortality is very low.

The responsible organism is *Bartonella bacilliformis,* discovered by Barton[5] in 1909. This microorganism can be observed in the red blood cells of the peripheral blood during the early stage of the disease, and it is also found by histologic examination of the eruptive lesions. Transmission of human bartonellosis is effected by means of Phlebotomus insects of which the species *verrucarum* appears to be the one chiefly responsible.[6]

CASE REPORTS

CASE 1.—A 21-year-old woman who came from a region where human bartonellosis is endemic was admitted to the Loayza Hospital (Lima) Aug. 31, 1944. Her symptoms began twenty-three days before when she suddenly developed general malaise and irregular fever. During the first few days of the illness she received quinine and atabrine because a presumptive diagnosis of malaria had been made, and six days before admission she was given a transfusion of 100 c.c. of whole blood. On admission the patient presented marked pallor with a generalized moderate icteric tinge; she was very restless, dyspneic, and appeared to be toxic. Her temperature was 39.7° C.

Physical examination revealed no significant abnormalities except for questionable splenic enlargement. An analysis of a venous blood sample made on the day of admission gave the following results: red blood cells, 780,000 per cubic millimeter; hemoglobin, 2.7 Gm. per 100 c.c.; leucocytes, 14,970 per cubic millimeter; hematocrit, 7.6 per cent; mean corpuscular volume, 97.0 cubic microns; mean corpuscular hemoglobin, 34.0 micro micro-

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Received for publication, Sept. 19, 1945.

grams; mean corpuscular hemoglobin concentration, 35.0 per cent; total, direct, and indirect plasma bilirubin, 2.39, 0.95, and 1.44 mg. per 100 c.c., respectively. The leucocytic formula was found to be: 1 per cent myeloblasts, 9 per cent myelocytes, 4 per cent metamyelocytes, 19 per cent stab neutrophiles, 44 per cent segmented neutrophiles, 3 per cent eosinophiles, 1 per cent basophiles, 6 per cent monocytes, and 13 per cent lymphocytes. The presence of numerous normoblasts and erythroblasts (61 and 11 per cent, respectively) was also noted and 43 per cent of the red blood cells were reticulocytes. In the examination of 1,000 red blood cells to ascertain the degree of parasitism, it was found that 94 per cent of them harbored *B. bacilliformis,* all of the bacillary (rodlike) type; the number of organisms in the parasitized erythrocytes varied from 1 to 6.

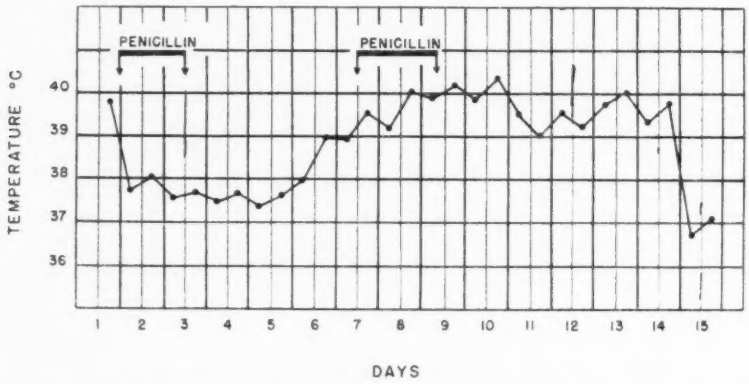

Fig. 1.—Case 1. Body temperature before and during the administration of penicillin.

The administration of penicillin* was initiated one day after admission. A total of 300,000 units was given in twelve doses of 25,000 units each every three hours. The first four doses were given intravenously, the remainder by intramuscular route. A second course of penicillin therapy was instituted seven days later; a total dose of 300,000 units was also given this time, divided into fifteen injections of 20,000 units each every three hours by intramuscular route. In Fig. 1 is shown graphically the body temperature of the patient before and during the period in which she received penicillin. About twenty-four hours after the initiation of this therapy, the temperature dropped to 37.8° C., and this level was approximately maintained during the next four days. There was an attendant marked improvement in the general condition of the patient; toxic manifestations disappeared and she was mentally quiet and relieved. On the fifth day the temperature rose again and remained on a high level for about nine days. A presumptive diagnosis of a secondary infection was not verified by laboratory investigations (blood culture, agglutina-

TABLE I. OBSERVATIONS IN CASE 1—RED BLOOD CELL COUNT AND DEGREE OF PARASITISM IN RELATION TO PENICILLIN ADMINISTRATION

SAMPLE	DATE	PENICILLIN THERAPY*	RED BLOOD CELLS (MILL. PER C.MM.)	PARASITISM† (PER CENT)
1	8/31/44 (9 A.M.)	None	0.78	94.3
2	9/ 1/44 (9 A.M.)	None	0.89	84.0
3	9/ 1/44 (4 P.M.)	None	—	92.6
4	9/ 1/44	6 hr. after first dose	—	95.4
5	9/ 2/44	12 hr. after first dose	0.96	82.0
6	9/ 2/44	18 hr. after first dose	—	66.0
7	9/ 2/44	24 hr. after first dose	—	20.6
8	9/ 3/44	48 hr. after first dose	1.17	10.2
9	9/ 4/44	72 hr. after first dose	1.51	8.9
10	9/ 5/44	96 hr. after first dose	1.52	3.9
11	9/ 6/44	120 hr. after first dose	1.32	0.7
12	9/ 7/44	144 hr. after first dose	1.61	0
13	9/ 8/44	168 hr. after first dose	—	0.3
14	9/ 9/44	192 hr. after first dose	2.22	0

*Penicillin was administered in two courses; the first one consisted of twelve doses of 25,000 Oxford units each every three hours (total 300,000 Oxford unit). The first dose was given six hours before sample 4. The second course was administered in fifteen doses of 20,000 units each every three hours in a total amount of 300,000 Oxford units. The first dose of this series was given immediately after analysis of sample 12.

†Parasitism means per cent of red blood cells parasited with *B. bacilliformis.*

*Trademark, Schenley.

tion tests for typhoid and paratyphoid, stool analysis, etc.). Two days after this rise of temperature the second administration of penicillin was begun; in addition to this, the patient received a blood transfusion of 900 c.c., 2 c.c. of liver extract intramuscularly, and small doses of vitamin C and thiamine chloride. The temperature fell by crisis on the tenth day and the patient remained afebrile.

The findings in regard to the red blood cell count and the degree of parasitism before, during, and after the period of penicillin therapy are summarized in Table I and are given graphically in Fig. 2. In three analyses made previous to the first dose of penicillin, the percentage of red blood cells parasited with *B. bacilliformis* varied between 84.0 and 94.3 per cent; twelve hours later, when the patient had received 125,000 units, the percentage fell to 82.0 per cent; and twenty-four hours after the initiation of the therapy, by which time 225,000 units of penicillin had been given, only 20.6 per cent of the red blood cells showed parasites. The degree of parasitism fell rapidly in succeeding days and no parasites were found in the blood on the sixth day after the first dose of penicillin.

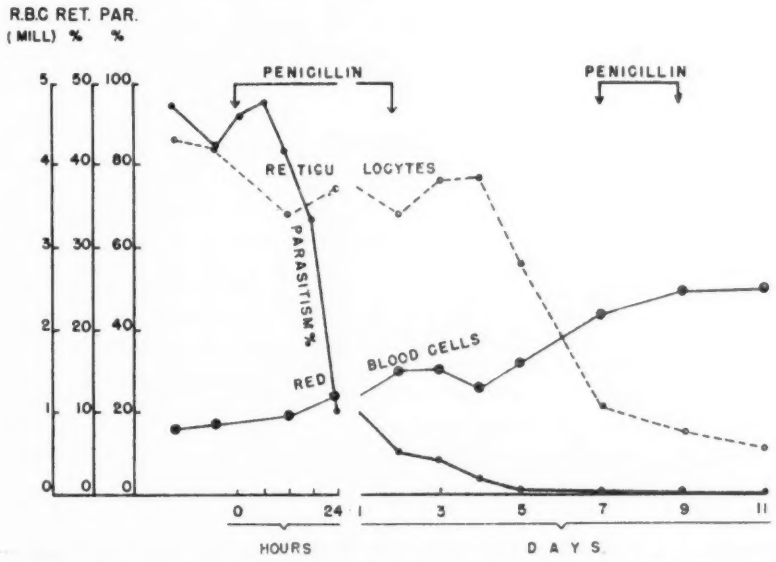

Fig. 2.—Case 1. Red blood cells, reticulocytes, and degree of parasitism before and during the administration of penicillin.

The morphologic characteristics of the *B. bacilliformis* showed marked changes during the penicillin therapy; previous to this, all the microorganisms were of the so-called bacillary type, in the form of small rods, with well-defined borders and a homogeneous red-violet color (Wright's stain used). In the blood smear made from a sample obtained six hours after the first penicillin injection, most of the microorganisms were of the coccoid type and appeared as small rounded bodies, quite similar to basophilic stipplings of the red blood cells. The bacillary type of bartonellae, which still remained in a few erythrocytes, showed ill-defined borders and a variable thickening; the staining characteristics were also altered with a loss of the homogeneous color. Some of the bacilli had only taken the stain at their ends so that there was a clear zone in the middle; some other bartonellae had assumed an annular shape resembling *Haemobartonella canis*. In the red blood cells which harbored several microorganisms, a definite tendency to conglomeration and a loss of the individual borders were noted. In the blood smears taken twelve and twenty-four hours after the initiation of the penicillin therapy, the morphologic changes just described were evident in all the observed microorganisms and, at this time, bacillary forms were no longer found. The absence of *B. bacilliformis* from the blood, which was evident on the sixth day of therapy, was maintained during the period in which the patient showed a secondary rise in temperature.

The gradual elevation in the number of red blood cells per cubic millimeter, which took place as the degree of parasitism decreased, can be observed in Table I and in Fig. 2. It is also interesting to notice in Fig. 2 that penicillin apparently did not interfere with the erythropoietic hyperactivity, as shown by the large numbers of reticulocytes in the peripheral blood during the time of its administration.

This patient has been observed during the last eleven months; her condition has remained good and at no time have the eruptive lesions, characteristic of the second stage of the disease, been observed.

CASE 2.—A 21-year-old man came to our office Dec. 11, 1944. He had been living for the previous six months in an area where human bartonellosis was endemic. His illness began eight days before with headache and chills, followed by an irregular type of fever. He also complained of pain on the right side of the neck and shoulder and of an intense feeling of malaise and weakness. The only positive findings in the clinical examination were a discrete subicteric tinge, a palpable spleen, and a few enlarged, nontender glands in the neck and inguinal regions. The temperature was 38.2° C. An analysis of a capillary blood sample, obtained on the same day, gave the following results: red blood cells, 4,320,000 per cubic millimeter; leucocytes, 8,100 per cubic millimeter; leucocytic formula, 28 per cent stab neutrophiles; 31 per cent segmented neutrophiles; 2 per cent eosinophiles; 16 per cent monocytes; and 23 per cent lymphocytes. Examination of the blood smear showed that 3.4 per cent of the red blood cells harbored *B. bacilliformis* of the bacillary type. A second blood analysis, made two days later, showed that the red blood cell count had come down to 3,630,000 per cubic millimeter; the leucocytes were 10,850 per cubic millimeter, and the percentage of red blood cells harboring *B. bacilliformis*, all of them of bacillary type, had increased to 31.3 per cent.

The administration of penicillin* was initiated forty-eight hours after the finding of *B. bacilliformis* in the peripheral blood. A total of 300,000 units was given in 15 doses of 20,000 units each every three hours; the first one was given intravenously and the rest of them by the intramuscular route. On the second day of the treatment the fever disappeared and there was a marked improvement in the clinical condition of the patient; the degree of parasitism at this time had decreased from 31.3 per cent of red blood cells parasited with *B. bacilliformis* to 6.8 per cent (Table II). Because of difficulties in obtaining penicillin, its administration was suspended for the next two days. This omission was followed by an aggravation of the patient's condition with a reappearance of the fever and a rapid increase in the degree of parasitism, which reached the high figure of 96.5 per cent of red blood cells with parasites. A further reduction in the number of red blood cells per cubic millimeter also occurred (Table II). Penicillin was again given every three hours intramuscularly in doses of 20,000 units each, up to a total of 800,000 units. In the sample of blood taken forty-

TABLE II. OBSERVATIONS IN CASE 2—RED BLOOD CELL COUNT AND DEGREE OF PARASITISM IN RELATION TO PENICILLIN ADMINISTRATION

SAMPLE	DATE	PENICILLIN THERAPY*	RED BLOOD CELLS (MILL. PER C.MM.)	PARASITISM† (PER CENT)
1	12/11/44	None	4.32	3.4
2	12/13/44	None	3.63	31.3
3	12/14/44	Penicillin administered	3.23	36.6
4	12/15/44	Penicillin administered	3.43	20.0
5	12/16/44	None	3.37	6.8
6	12/17/44	None	2.98	44.6
7	12/18/44	None	2.69	96.5
8	12/20/44	Penicillin administered	1.73	90.2
9	12/21/44	Penicillin administered	1.92	63.2
10	12/22/44	Penicillin administered	2.12	36.2
11	12/23/44	Penicillin administered	1.92	6.7
12	12/24/44	Penicillin administered	2.15	2.0
13	12/26/44	None	2.40	0.9
14	12/27/44	None	2.28	0
15	12/29/44	None	2.11	0
16	12/31/44	None	1.89	0
17	1/ 3/45	None	2.31	0
18	1/ 8/45	None	2.70	0
19	1/13/45	None	3.01	0
20	1/24/45	None	3.60	0
21	2/20/45	None	4.49	0
22	6/24/45	None	4.84	0
23	8/27/45	None	5.34	0

*Penicillin was administered in two series, 300,000 Oxford units and 800,000 Oxford units total doses, respectively. The individual doses were 20,000 units every three hours. The first series started nineteen hours before sample 3; second series was begun forty-three hours before sample 8.

†Parasitism means per cent of red blood cells parasited with *B. bacilliformis*.

*Trademark, Schenley.

three hours after the initiation of this second series of penicillin injections, a decrease in the degree of parasitism was observed. This change became more accentuated in successive days, although the rate of disappearance of the parasitism was slower than in Case 1. No bartonellae were found in the blood two days after the last dose of penicillin.

The changes in the morphologic characteristics of the microorganisms, observed during the period of penicillin therapy, were quite similar to those observed in Case 1. About twenty-four hours after its initiation the microorganism changed its shape from the bacillary to the coccoid type and presented the staining alterations already described. It was interesting to observe that after the first series of penicillin injections had been completed, simultaneous with the relapse, a large number of microorganisms assumed again the bacillary shape with well-defined borders and a uniform color. The reinstitution of penicillin therapy, which was accompanied by the reduction in the percentage of parasited red blood cells with *B. bacilliformis,* again resulted in the morphologic variations of the type already described.

This patient has been examined on three different occasions since recovery, the last one being after about eight months. He reported that about three months after leaving the hospital he noticed a single small nodular lesion in the right gluteal region; however, no signs of this eruption could be found in an examination made one month after the reported appearance of this lesion. His general condition has remained good.

COMMENT

The various antiparasitic, therapeutic procedures employed so far in the treatment of human bartonellosis have not proved to be satisfactory. The product Sdt 386 B, containing 18 per cent arsenic and 20 per cent antimony, which is highly effective in controlling *Haemobartonella muris* infection in rats, has been used by Manrique and Rocha[7] in the treatment of fourteen cases of Carrion's disease; the opinion of these authors, who concluded that the use of the drug is beneficial, is not shared by others. Quinine, atabrine, different arsenic preparations, and sulfonamide derivatives have also been used without success. Roca[8] has lately recommended the use of a glycerin preparation and has reported a favorable action, but his findings have not been confirmed by other workers. Immunologic therapy, using sera prepared in animals, has been recently investigated by Howe,[9] but the results observed do not as yet permit a definite evaluation. Whole blood transfusions have not been widely employed as a therapeutic measure; one of the patients reported in this paper received 900 c.c. of whole blood without untoward effects.

It is evident that evaluation of the effectiveness of penicillin in the treatment of human bartonellosis can be made only after a prolonged trial, especially if we take into consideration that spontaneous recovery does occur in a large number of cases, even in those patients who show a severe degree of anemia and toxicity. It must also be remembered that secondary infections develop frequently during the first stage of the illness and, in the opinion of most observers, are in part responsible for the high degree of mortality. In these complicated cases penicillin administration may not prove to be satisfactory if the organism responsible for the associated infection belongs to a type which is refractory to the action of this drug. Cachay[10] has recently reported the death of an infant, with a severe degree of anemia due to human bartonellosis, who was treated with large doses of penicillin during the twenty-four hours previous to his death.

The difficulties of transmitting human bartonellosis to animals decrease the possibilities of a prompt evaluation of this therapeutic problem. Ubatuba and Vieira,[11] in Brazil, have observed negative results in the penicillin treatment of *H. muris* infection in rats. From the standpoint of human bartonellosis, the value of this experiment is, however, limited if it is remembered that the product Sdt 386 B, with a very uncertain action on *B. bacilliformis,* is, on the contrary, highly effective in controlling *H. muris* infection in rats. Because of

the similarities which have been noted to exist between the anatomical lesions produced by *B. bacilliformis* infection and those caused by the presence of Rickettsia, it is interesting to mention that Moragues, Pinkerton, and Greiff[12] observed that penicillin inhibited, in vitro, the growth of Rickettsia cultures in chick embryos and also protected rats against the development of infection with this organism.

In our opinion, the most promising aspects of the results observed in the two cases here reported are the decrease in the percentage of parasited red blood cells with *B. bacilliformis* and the very definite morphologic changes which took place in these microorganisms, carried by circulating red blood cells, within the first twenty-four hours after penicillin administration. Such changes, when found in untreated patients who recover spontaneously, do take place in a longer time and at a slower rate. Delgado[13] has recently observed a rapid decrease in the degree of parasitism and in the morphologic characteristics of the *B. bacilliformis* (changes similar to those described by us) in a patient with bartonellosis with a severe degree of anemia, who recovered after receiving large doses of penicillin.

It will be important to study in the future the effect of penicillin on the development of the eruptive lesions of human bartonellosis; Vila Acuña[14] has reported a prompt regression of cutaneous nodules in a patient treated with this drug. It will also be necessary to determine whether these lesions fail to develop in patients who have received penicillin therapy during the first stage of the illness; such occurrence, noted at least in one of our two patients, would favor the interpretation that sterilization of *B. bacilliformis* is accomplished during the period of therapy. Aldana,[15] who is now studying the effect of penicillin in vitro, is of the opinion that this drug has a bacteriolytic effect upon cultures of *B. bacilliformis*.

SUMMARY

Penicillin was employed in the treatment of two patients with bartonellosis (Carrion's disease) during the initial noneruptive period of the illness.

The favorable effects observed clinically in these patients and in the degree of red blood cell parasitism, together with the changes in the morphologic characteristics of the responsible organism in the red blood cells, suggest the desirability of carrying out further studies to elucidate the value of penicillin in the treatment of human bartonellosis.

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A STANDARDIZED BACK BURN PROCEDURE FOR THE WHITE RAT SUITABLE FOR THE STUDY OF THE EFFECTS OF THERAPEUTIC AND TOXIC AGENTS ON LONG-TERM SURVIVAL

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WITH THE TECHNICAL ASSISTANCE OF JANE R. LEWIS AND JEAN CONVER

IN ORDER to evaluate various methods of therapy in the treatment of severe thermal burns in rats, as evidenced by long-term survival, a standard burn procedure has been developed with the following objectives: (1) reproducible survival rates; (2) preservation of uninjured extremities, thus allowing the animal greater freedom of movement and the investigator untraumatized veins for infusion and hematocrit sampling; (3) elimination of complications from additional trauma and hemorrhage resulting from autoingestion; and (4) preservation of uninjured body orifices to permit voiding of urine and defecation.

The Experimental Animal.—The Wistar rat* was used as the test animal. All rats received from the Wistar Institute were kept in our stock room for at least one week prior to their use. The animals were kept in small cages (five per cage) and all were fed Purina dog checkers supplemented with whole milk. Approximately twenty-four hours prior to an experiment, the desired number of rats of the proper weight range was selected at random from the stock cages and moved into the experimental room. They were kept in individual wire cages at an average room temperature of 27° C. (variation 26 to 30° C.) and an average relative humidity of 52 per cent for the duration of the experiment. Diurnal variations in temperature fell within the above range; however, because of lack of air-conditioning facilities, diurnal humidity variation was uncontrolled. This temperature was found by Horst, Mendel, and Benedict[1] to be that at which the minimum metabolic rate or a state of thermal equilibrium prevails. The rats had free access to food and water until about one hour prior to burning, at which time they were reweighed and carefully examined for any signs of respiratory infection. All animals exhibiting a questionable infection were discarded.

Observations on several groups of burned rats having free access to food and water showed great individual variation with respect to water intake during the first twelve hours after the burn. During this interval some rats did not drink at all. During the second twelve-hour interval following burning all rats drank some water. Hence, in order to lessen any probable effect resulting from the individual variation, water was withheld from all burned rats for the first twelve hours following the burn. Since very few animals showed any interest in food during the first twenty-four hours postburn, food was therefore withheld during this period.

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Work done under contract with the Office of Scientific Research and Development.

Received for publication, Aug. 16, 1945.

*For reasons of availability, the rats used in these experiments were those not used for breeding because of their failure to conform to growth rate standards. The rats were otherwise healthy animals.

Technique and Conditions of the Burn Procedure.—A scald burn of the back was produced by immersion in a constant temperature bath (Fig. 1). The unshaved rats were anesthetized in a glass specimen jar, volume 8,816 c.c., containing a coarse mesh wire screen platform elevated 9.0 cm. over a pad of ether-saturated cotton (Fig. 1). Optimum anesthetic conditions were obtained

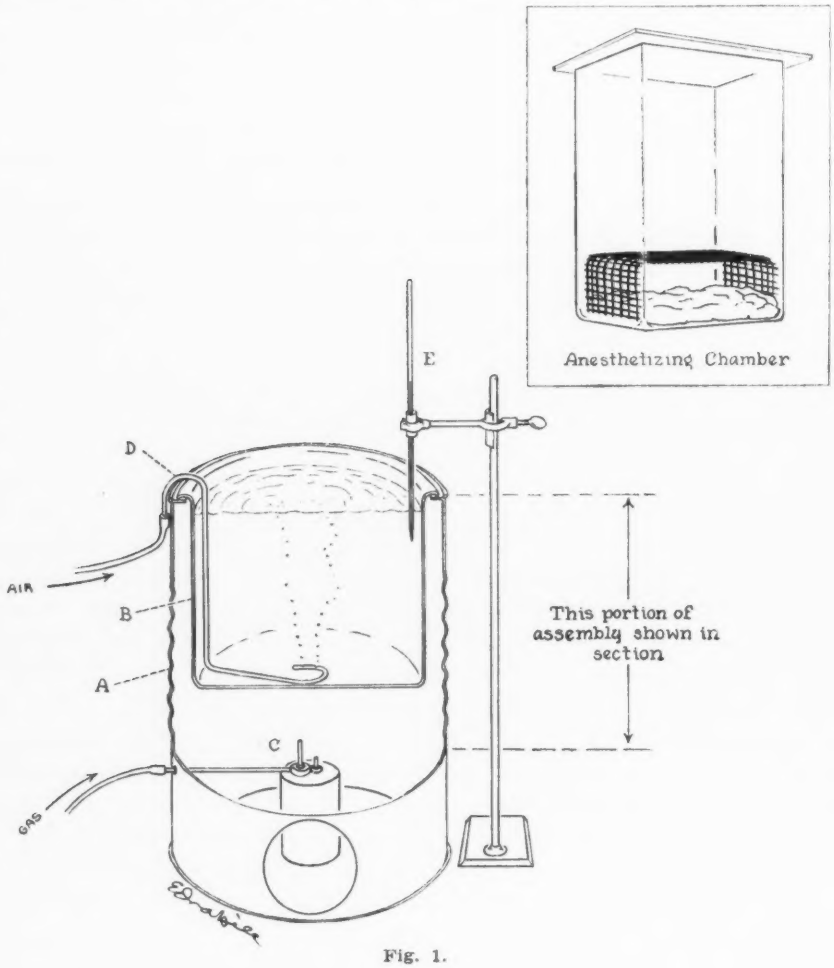

Fig. 1.

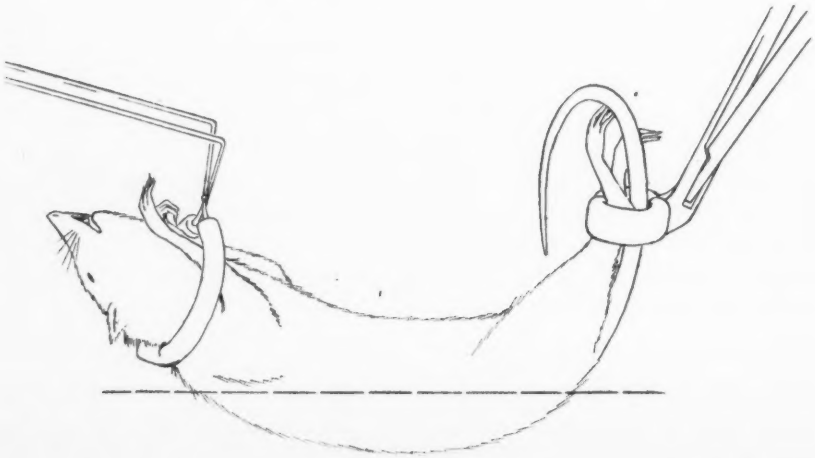

Fig. 2.

when the ether concentration was such as to produce complete anesthesia within 90 to 120 seconds. The forefeet (bound together by a rubber band) and head were held in a bone forceps (Fig. 2). The rats were immersed for fifteen seconds, timed by a stopwatch, at a bath temperature of 90° C. ± 0.25° C. The bath temperature was recorded by a mercury thermometer lowered to a depth of 5.0 cm. below the surface of the bath. The water was continuously agitated by a compressed air bubbler placed on the bottom of the tank (Fig. 1). When removed from the bath, the rats were gently blotted by rolling them in an absorbent towel and then removed to individual wire cages.

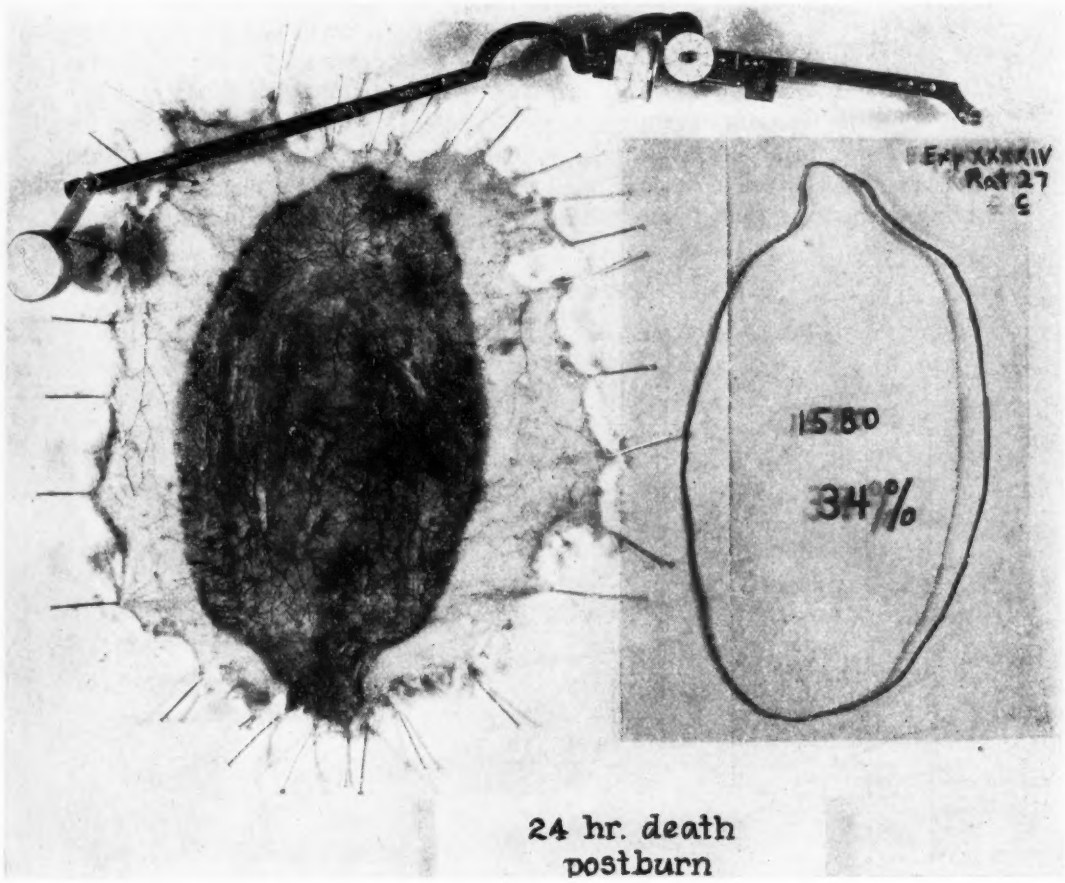

Fig. 3.

Measurement of, and Limits of Variation of, the Burned Area.—A series of unshaved rats were immersed from the base of the skull to the base of the tail, with the lateral and ventral burned area varied by arching the back. Examination of the internal surfaces of the skin of these burned rats showed the burned area to be sharply demarcated from the uninjured area. The skins were smoothed out just enough to allow them to lie flat when pinned to a flat cork surface. The perimeter of the burned area was traced on a piece of cleared x-ray film with a glass marking pencil and the tracing measured by a planimeter (Fig. 3). The relationship of the burned area to the unburned area was expressed in terms of per cent of the total body surface, which was calculated from the formula $S = KW^{0.60}$ ($K = 12.54$), given by Lee[2] for the white rat.

By varying the curvature of the back on immersion from that in which the belly is covered by water to that in which the water covers only the dorsal surface, the burned area can be varied from 45 per cent to 10 per cent of the total body surface without involving the extremities or the tail. It was found that with practice, using anatomical landmarks, any desired per cent surface within the above range could be reproduced within ± 2 per cent. The survival data reported below has been obtained for rats receiving burns of 20 per cent, 32 per cent, 36 per cent, and 45 per cent, with a maximum variation of ± 2.

Skin measurements on three groups each of four and eight rats sacrificed at twenty-four hours following the burn showed a consistent variation in per cent surface burned of ± 2. However, in all experimental groups in which the survival rates were checked for ten days, the burned areas of the surviving rats sacrificed at ten days were found consistently to average about 6 per cent less than those in the same groups dying within seventy-two hours following the burn. Hence, in all experimental groups two checks on the uniformity of the burned surfaces were obtained. All animals within any experimental group having burned areas not falling within the desired range were eliminated.

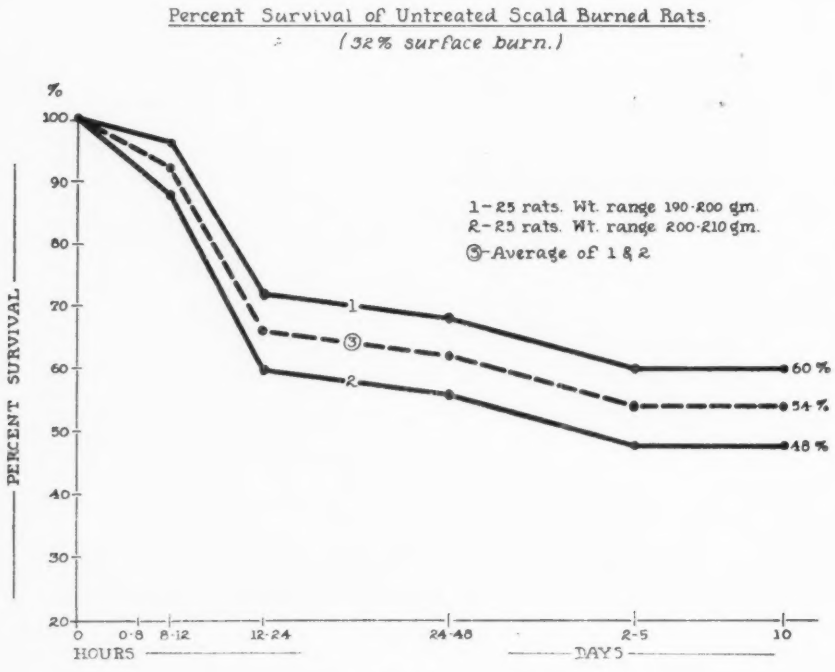

Fig. 4.

The Relationship of the Burned Surface to Survival.—Preliminary experiments showed that in order to obtain reproducible survival percentages for any given per cent burn administered according to the conditions outlined, the initial weights of the burned rats must fall within a closely selected weight band. Accordingly, two weight bands have been studied extensively: (1) 190 to 200 grams and (2) 200 to 210 grams. Within these two weight groups, all rats receiving burns of 35 per cent or more of their total body surface have died within forty-eight hours following the burn (ten rats in each weight group), and all rats receiving burns of 20 per cent or less of their total body surface have survived (ten rats in each weight group). Those animals receiv-

ing a burn between 30 and 34 per cent of their total body surface, produced by immersion from the seventh cervical vertebra to the first caudal vertebra and to such a depth that a lateral line would pass through these two points, the glenovertebral angle of the scapula and the third trochanter of the femur, have shown a consistent average survival percentage for the combined weight groups of 54 per cent. In Fig. 4 is shown the per cent survival of twenty-five rats in each weight group and the average survival of the combined groups. The difference in per cent survival between these two weight groups is not statistically significant; however, because of the variation, equal numbers of animals in the two weight groups were used in all experiments.

Relationship of Sex to Survival.—Both male and female rats were tested under the standard conditions. No significant difference in survival was found. Consequently, both male and female rats were used in approximately equal numbers.

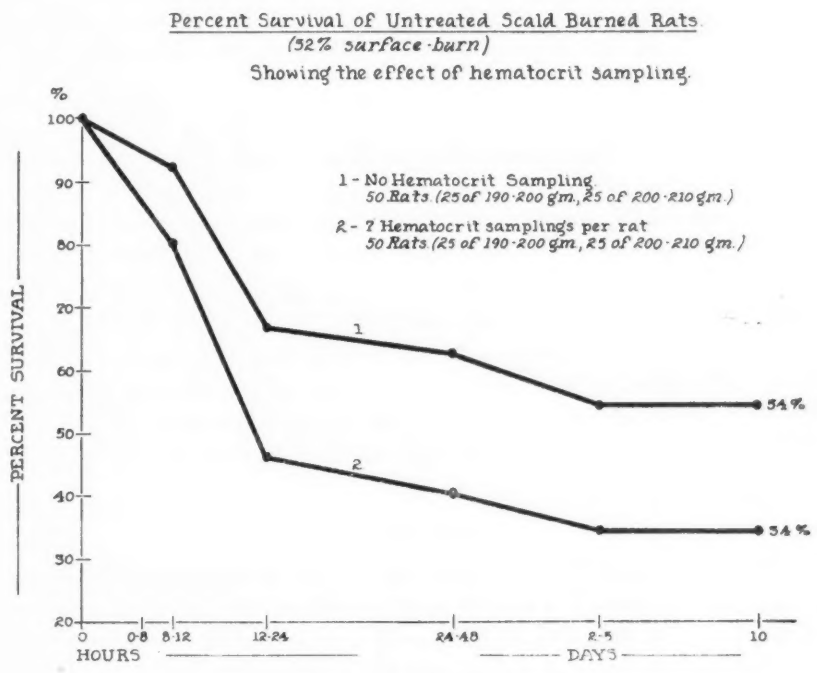

Fig. 5.

Effect of Manipulation on Survival.—Early analysis of the data showed that the handling of burned rats and the resultant blood loss, although small, in taking hematocrit samples markedly influenced survival. These samples, seven from each rat, were obtained by loosely rolling the unanesthetized animal in a towel, nicking the lateral tail vein, and drawing up 0.01 c.c. of blood into a uniform bore capillary tube, after which the wound was closed by applying pressure through a sterile gauze sponge and by sealing with a drop of collodion. In Fig 5 is shown a significant decrease in survival between the fifty unhandled animals, curve 1, and the fifty animals subjected to hematocrit sampling represented in curve 2 corresponding to a probability value of between 0.02 and 0.01 as calculated from the Chi square formula of Fisher.[3]

Effect on Survival Resulting From Various Deviations From the Standard Procedure.—The survival rates recorded in Figs. 4 and 5 for the 32 per cent burn were reproducible only when all variable factors were rigidly controlled.

Four or more animals were used in each experimental group and in no experimental group included in the data given did all of the animals survive or die when the standard conditions obtained. Hence, small changes in the standard conditions described below which caused all of the four or more of the tested animals either to survive or to die were considered real, although the numbers of tested animals were too small to be treated statistically.

With all other factors constant, a lowering of the bath temperature of 2° C., that is, 88° ± 0.25° C. for fifteen seconds, permitted 100 per cent survival of ten rats tested.

A reduction of the immersion time from fifteen to ten seconds, other factors constant, allowed a 100 per cent survival of ten rats tested. Increasing the immersion time from fifteen to twenty seconds reduced the survival of ten rats tested to 20 per cent.

Fifteen larger animals weighing between 220 and 230 grams when subjected to the standard burn conditions all survived. However, with other conditions standard, all rats ranging in weight from 220 to 350 grams died when given a 45 per cent ±2 body surface burn.

The experiments giving the survival rates recorded in Figs. 4 and 5 were done in the months from November to May, inclusive. In June, however, twenty-one of twenty-two (95.5 per cent) rats tested survived. These experiments were done with a mean elevation in room temperature of 2° C. and an increase in average humidity from 52 to 62 per cent. All other experimental conditions were maintained as outlined in the standard procedure. The survival rate of these twenty-two animals when compared to that of any group of twenty-two of the 100 rats reported in Figs. 4 and 5 shows a statistically significant difference. Other factors than those of temperature and humidity, for example, seasonal variations in quantity and character of the fur and seasonal blood volume changes, may be responsible for the increased survival rate.

DISCUSSION

The back burn procedure outlined has several advantages over the complete immersion type of burn for a particular portion of the body, especially when it is desired to maintain the animals over a long period of time postburn for study. The animals are not able to eat the injured tissue, thus avoiding additional trauma and hemorrhage. The extremities remain functional, allowing the animal freedom of movement and allowing the investigator untraumatized veins for infusion and blood-sampling procedures.

It was found that rats burned to the axillas were usually unable to void urine or defecate normally after seventy-two hours following the burn as a result of scab formation on the external genitals and anus. These tissues are not affected as a result of the back burn and hence no complications of this nature arise.

Intraperitoneal complications arising when the ventral body surface was burned were also avoided by the back burn.

Since the burned area can be easily removed upon the death or sacrifice of the animal, accurate measurements of it can be made, thus permitting comparisons of uniformly injured surface areas.

From the data presented it can be concluded that the reproducibility of the recorded survival percentages is critically dependent on the standardization of all conditions incident to the method. Although other strains of rats were not tested, it is possible that the data recorded for this procedure may not be reproducible for other strains of rats.*

SUMMARY

A standard back burn procedure for the white rat (Wistar strain) is described. Survival rates are recorded for the following percentages of the total body surface burned: 20 per cent, 32 per cent, and 45 per cent (all ± 2). Environmental and bath temperature changes, humidity variations, seasonal change, variability in immersion time, animal variables including strain, weight, sex, and condition, and manipulations to which the animals may be subjected are all discussed in relation to their effect on survival.

Because of the sensitivity of the method to changes in any one of several variable factors, it is recommended that simultaneous controls always accompany experimental groups.

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*Subsequent work done on Wistar rats from the breeding stock since this paper went to press has shown that in order to obtain survival rates similar to those reported, it was necessary to increase immersion time while keeping all other factors constant. Fifty per cent survival was obtained by immersing for twenty seconds. A 100 per cent mortality was obtained by immersing for thirty-five seconds.

HYDROLYZED CASEIN (CAPAIN) AS A PLASMA SUBSTITUTE

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THE continuing attempts to find a suitable plasma substitute indicate, since the use of Bayliss' gum solution, its need in many cases of surgical shock and allied conditions. In order to find a plasma substitute which combines a considerable water-binding power with complete tolerability, we manufactured a protein hydrolyzate from casein by papain, "capain," showing the following properties:

1. The hydrolyzate is prepared by digesting commercial casein in buffer solution (pH = 5.8) with unactivated papain at 70° C. All unhydrolyzed protein in the resulting mixture is precipitated by adding trichloroacetic acid up to a final concentration of 3 per cent. The trichloroacetic acid is removed by boiling at slightly alkaline reaction, the pH is adjusted, and the solution is concentrated to the desired strength, usually to a nitrogen content corresponding to a 15 per cent protein solution. A suitable preservative may be added. The capain solution is sterilized by boiling once more and kept in sealed glass bottles; it can be stored for over a year (or longer) without cloudiness or other interferences with suitability.

2. Preliminary tests have shown, that although free amino acids may occur, most of the hydrolyzate is in the form of polypeptides, the average degree of hydrolysis having proceeded to the octo-peptide stage. Tests for important amino acids are all strongly positive, especially so when a small amount of lactalbumin had been added to the casein before digestion.

In Fig. 1 is illustrated the water-attracting power of a 13 per cent capain solution compared with that of 2 and 5 per cent sodium chloride solutions, to 10 per cent glucose, and to human plasma. Care has been taken that not only the initial but also the prolonged attraction of water through cellophane was tested. It is seen that the oncotic attraction of the capain solution is about seven times the attraction by plasma; this can also be observed by setting capain and plasma against each other in an osmometer. Capain will exert a considerable osmotic pressure (more than 2 M. of water) against plasma and uphold it for ten hours or more.

The strength of a capain solution may be evaluated by a Kjeldahl nitrogen estimation or by polarimetric or osmometric measurement. All three kinds of determination give proportional results.

The cellophane ultrafiltrate of capain contains about one-third of its nitrogen content, so roughly two-thirds of the nitrogenous material does not pass through cellophane. The depression of the freezing point of capain ultrafiltrate is -0.85° C., so it must be considered as slightly hypertonic.

3. We can make the following statements about the pharmacology and immunology of capain injections:

(a) In rabbits, capain was very effective in restoring the blood pressure lowered by extensive bleeding to such degree that the infusions of salt solutions

From the Biochemical Institute and Surgical Clinic.
Received for publication, Aug. 23, 1945.

could not prevent severe hypotension. In normal animals injections of a large amount of capain (2 c.c. per kilogram) caused a slight increase in blood pressure; no other symptoms were observed.

It was not possible to sensitize rabbits by previous injections of capain in the usual way; no anaphylactic or cutaneous reactions developed and no precipitin was formed. We consider, however, that the testing of a solution for the treatment of shock cannot be carried out by animal experiments alone; the human reaction is so much more sensitive that in our opinion the final evaluation can be made only on human subjects (recorded under 3 [b]).

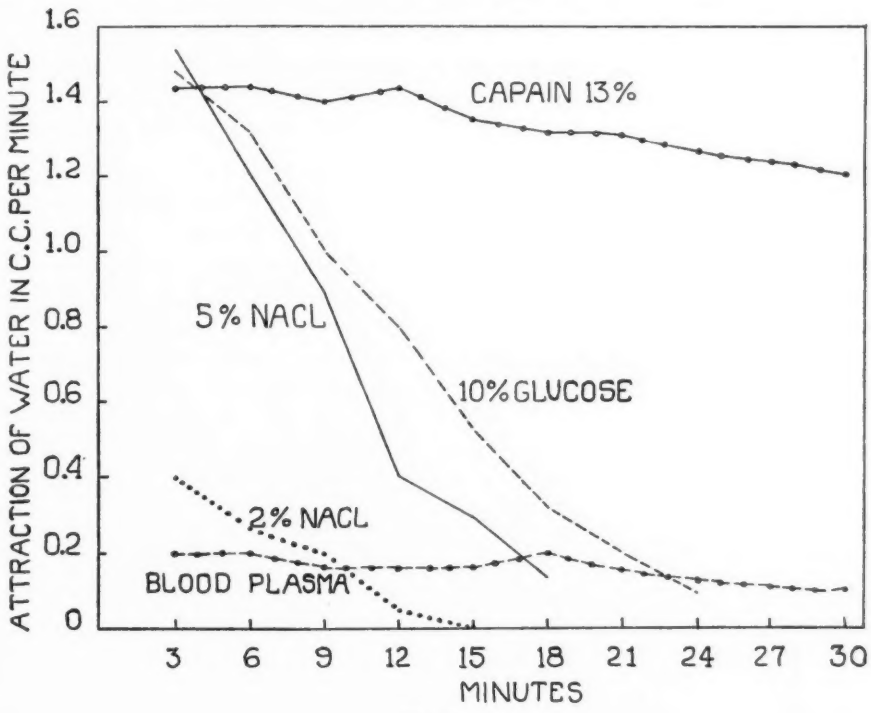

Fig. 1.

(b) Capain was given intravenously in increasing doses up to 20 c.c. to normal persons and to suitable patients; no reactions, with the exception of slight headache and facial heat, were observed; any cutaneous reactive sensitivity to capain was absent. Capain infusion was given in the umbilical veins in prematurely born infants, with a view to provide protein feeding; no food was given orally. It was shown by Dr. J. Jonxis, in the Rotterdam pediatric clinic, that a positive nitrogen balance could be maintained in this way. Also in the newborn infant and in older children the suitability of this hydrolyzate for intravenous protein nutrition was demonstrated. We consider these observations as better proofs for the nontoxicity of capain infusions in human patients.

In the Groningen pediatric clinic a number of starved children (about forty) with "infectious toxic shock" symptoms was successfully treated (in regard to the shock symptoms) by capain infusions. This is another indication that, in the treatment of shock by capain infusions, toxic effects are not to be expected.

The possibility of protein growth from the intravenous injection of a casein digest not completely hydrolyzed may be in accordance with the assump-

tion of Madden and Whipple[1] and others who showed that protein-starved dogs can be kept in nitrogen equilibrium by the intravenous injection of dog plasma without detectable increase in blood amino acid concentration. The hypothesis of realignment of larger polypeptides for intracellular protein synthesis without complete breakdown to amino acids is sustained by the experiments with tagged amino acids and by the observation of Fischer,[2] showing that protein growth in tissue cultures can in many cases be better maintained by a protein hydrolyzate in which the enzymatic breakdown has not progressed too far.

(c) Next to the water-attracting property of capain, a vasoconstrictor activity must be reckoned with. Arteriolar constriction was directly observed and filmed in a Lewis chamber in the rabbit's ear; its onset regularly was ten minutes after the intravenous injection of capain. Moderate venoconstriction not interfering with the infusion was seen in several cases by Dr. Jonxis during injection in an exposed vein in newborn infants.

4. Clinical experiences. The infusion of a solution containing capain for the treatment of various kinds of surgical circulatory shock was studied in the Groningen surgical clinic in seventy cases and was reported by one of us (L. A. G. H.) in the Communications of this Clinic, Vol. 5, p. 242. The treatment was uniformly successful in regard to the re-establishment of normal minute volume and arterial blood pressure, so that it has now become a routine procedure. In a large number of comparable cases outside the Groningen clinic, which we have not observed, the general result appears to have been the same. A comparison with whole blood could be made in about fifteen patients, and the result is certainly not unfavorable toward the capain infusion. In patients with a very low hemoglobin content whole blood is, of course, indicated.

A certain number of chills was observed (about ten), especially during very rapid injections. They were, as a rule, not severe and in every instance promptly responded to treatment; other deleterious effects were not present. Thrombosis was not observed, although the infusion was sometimes prolonged for from two to three days. In some cases the vasomotor action was clinically evident by a quick response of the blood pressure before the circulatory volume could have increased sufficiently.

The routine procedure in nearly all cases of surgical shock, characterized by low venous pressure, was rapid infusion of saline or glucose solution, to which 100 c.c. of capain per liter were added; in prolonged infusions the capain concentration may be lowered. The increase in both arterial pressure and venous filling must guide the rate of capain infusion; in severe cases it cannot be given too rapidly. The average total amount of capain per patient was from 200 to 300 c.c.; in extreme conditions, a total up to 800 c.c. has been given.

Capain is much simpler to store and administer than is plasma; it keeps indefinitely at room temperature, may be boiled, and is used by simply pouring into the saline infusion. This must be an advantage in field conditions, especially so in the Tropics.

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STUDIES ON HYPERTENSION

V. Effect of High Spinal Anesthesia on the Blood Pressure of Patients With Hypertension and Far-Advanced Renal Disease—Its Possible Relationship to the Pathogenesis of Hypertension

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Galveston, Texas

PREVIOUS investigations[1-4] have led us to doubt that essential hypertension is due to an abnormality of the renal pressor mechanism—renin, renin substrate, and angiotonin. This doubt resulted from studies of the effect of spinal anesthesia on the blood pressure of patients with essential hypertension, the effects of various agents including angiotonin on the blood pressure of these patients at the time of the fall produced by spinal anesthesia, and the bioassay for angiotonin in blood from normal patients, those with hypertension, and others made hypertensive by intravenous injection of angiotonin. In general, the patients previously studied had essentially normal renal function or at least did not have nitrogen retention.

Proponents of the humoral etiology of essential hypertension[5] have attempted to explain the considerable and long-lasting fall in blood pressure which may be caused by various operations on the sympathetic nervous system on the basis of a decrease in vasomotor tonus to the kidney alone. This is postulated to result in improvement of the renal blood flow and a consequent decrease in the production of renin by the kidney. While we have called attention to the unlikelihood of this in previous publications[2, 4] based on the studies mentioned, we believe that additional information bearing on this point might result from a comparison of the effects of high spinal anesthesia on the blood pressure of patients with hypertension and normal renal function with the effects similarly obtained on patients with hypertension and far-advanced renal arteriolar disease as shown by the presence of uremia.

Since the present study was undertaken, Corcoran and Page[6] have shown that there is no increase in diodrast and inulin clearances in patients whose blood pressures have been caused to fall by surgery on the sympathetic nervous system. Our studies[7] have shown clearly that there is a decrease in inulin and diodrast clearances in patients whose blood pressure has been caused to fall by spinal anesthesia.

The present study is particularly pertinent in view of Corcoran's[8] statement, "It is only those (patients with hypertension) in whom organic arteriolar disease is absent or minimal who consistently show evidences of vasomotor origin." It must be agreed that patients who have uremia resulting from essential hypertension have "far-advanced arteriolar disease," at least in the kidneys.

It is for these reasons that these studies have been made on patients with uremia. Our original approach in this direction was dictated by our belief that the elevated blood pressure of hypertensive patients with far-advanced renal arteriolar disease would not fall as much under spinal anesthesia as the

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Received for publication, Aug. 23, 1945.

TABLE I. EFFECT OF SPINAL ANESTHESIA WITH 150

PATIENT	SEX	AGE (YR.)	N.P.N. (MG. %)	UREA NITROGEN (MG. %)	MAXIMUM CONCENTRATION TEST	BLOOD PRESSURE BEFORE SPINAL ANESTHESIA	BLOOD PRESSURE DURING SPINAL ANESTHESIA†	DURATION OF SIGNIFICANT BLOOD PRESSURE FALL (MIN.)	LEVEL OF SENSORY ANESTHESIA	MOTOR PARALYSIS LOWER LIMBS	MALIGNANT PHASE	URINARY FINDINGS
S. B.	M	38	75	63	1.018	243/190	120/90	60	Slightly above nipple line	Yes	Yes	Albumin, 3+; gross blood; many casts
L. S.	F	44	120	75	1.018	226/108	124/70	76	2nd I.S.	Yes	Yes	Albumin, 2+; 4 to 5 R.B.C. per H.P.F. occasional cast
R. M.	F	46	81	51	1.012	240/142	126/80	37	2nd I.S.	Yes	No	Albumin, 4+; many pus cells and casts
C. S.	M	44	171	116		244/160	162/138	42	2nd I.S.	Yes	No	Albumin, 4+; many R.B.C. and pus cells; occasional cast
E. F.	F	49	123	90	1.009	242/124	75/50	86	Unable to cooperate	Yes	Yes	Albumin, 3+; frequent R.B.C., W.B.C., and casts
L. L.	M	54	235		1.026	218/108	186/90	53	2nd I.S.	Yes	No	Albumin, 3+; gross blood; 3 to 5 W.B.C. per H.P.F.; occasional cast
A. B.	F	21	81		1.012	250/164	220/146	49	3rd I.S.	Yes	No	Albumin, 3+; numerous pus cells and casts
E. H.	F	40	200	75	1.017	178/100	160/72	63	3rd I.S.	Yes	No	Albumin, 4+; many W.B.C.; occasional R.B.C.
T. D.	F	52	120	64	1.007	234/124	210/98	57	Clavicle	Yes	No	Albumin, 4+; frequent casts, R.B.C., and W.B.C.
W. C.	M	65	85	60	1.015	226/134	186/102	34	Xiphoid	Yes	No	Albumin, 4+; 8 to 10 R.B.C. per H.P.F.
H. R.*	M	44	300	202	1.009	202/128	186/108	43	Xiphoid	Yes	No	Albumin, 2+; many R.B.C., W.B.C., and casts
H. R.*	M	44	300	202	1.009	228/138	184/112	72	2nd I.S.	Yes	No	Albumin, 2+; many R.B.C., W.B.C., and casts
T. R.	M	34	200	140	Not done	178/100	156/100	80	Slightly above xiphoid	Yes	No	Anuria; albumin, 4+; many R.B.C., W.B.C., and casts

*Same patient.
†Lowest level of fall.

MG. OF PROCAINE HYDROCHLORIDE ON BLOOD PRESSURE

OCULAR FUNDI	ELECTROCARDIOGRAM	NECROPSY FINDINGS	REMARKS
Usual hypertensive changes; many hemorrhages and exudates	T_1, diphasic; T_2 and T_3, neg.	Not done	R.B.C., 3.2 M; Hb., 60%
Papilledema, hemorrhages, and exudates	S-T_1 and S-T_2, depressed; T_1 and T_2, neg.	Arteriolar nephrosclerosis	R.B.C., 3.5 M; Hb., 66%
Papilledema, hemorrhages, and exudates	Slurred QRS; T_1, T_2, and T_4, neg.; S-T_1 and S-T_2, depressed	Did not die while observed	R.B.C., 3.2 M; Hb., 62%
Usual hypertensive changes plus old exudate and hemorrhage	QRS slurred; T_1, low; T_4, neg.	Chronic pyelonephritis, bilateral and arteriolar nephrosclerosis	R.B.C., 2.9 M; Hb., 60%
Papilledema, hemorrhages, and exudates	S-T_2 and S-T_3, depressed; T_2, T_3, and T_4, diphasic	Not done	R.B.C., 2.8 M; Hb., 58%
Hemorrhages, exudates, and papilledema	Myocardial damage	Chronic glomerulonephritis with acute exacerbation	R.B.C., 2.5 M; Hb., 53%; hyperglobulinemia: 6.6 Gm. % globulin; 3.2 Gm. % albumin
Usual hypertensive changes plus hemorrhages and exudates	No myocardial damage	Arteriolar nephrosclerosis plus pyelonephritis	Died at home; kidney removed; R.B.C., 3.0 M; Hb., 58%
Advanced vascular changes	T_1, T_2 and T_4, diphasic; T_3, neg.	None	Old record indicates evidence of pyelonephritis; pyuria; back pain; R.B.C., 1.54 M; Hb., 38%
Usual hypertensive changes plus hemorrhages and exudates	S-T_1 and S-T_2, depressed; T_1, neg.	Arteriolar nephrosclerosis	R.B.C., 2.7 M; Hb., 50%
Old hemorrhages and usual hypertensive changes and exudates	QRS slurred; T_1 and T_4, neg.	Arteriolar nephrosclerosis	No anemia
Usual hypertensive changes, hemorrhages, and exudates	Not done	Chronic glomerulonephritis	R.B.C., 1.5 M; Hb., 25%
Usual hypertensive changes, hemorrhages and exudates	Not done	Chronic glomerulonephritis	R.B.C., 1.5 M; Hb., 25%
No report	Not done	Patient recovered	Diagnosis: Acute glomerulonephritis; R.B.C., 3.8 M; Hb., 77%

blood pressure of patients with hypertension and normal renal function under the same conditions if the initial blood pressure elevation is due to a humoral substance produced in the kidney. It is logical to assume that interruption of vasomotor impulses to the kidney is not likely to influence favorably the circulation through the kidney which has such extensive and intensive vascular pathology as to result in uremia; and it is pertinent again, in this relationship, to refer to work[7] which has demonstrated a decrease in inulin and diodrast clearances in hypertensive patients whose blood pressure has been caused to fall to normal by interrupting vasomotor function with spinal anesthesia.

METHODS

Hypertensive patients with uremia were selected at random. Preliminary control blood pressures and experimental observations of the effect of spinal anesthesia on the blood pressure levels were done as previously described.[1] All of the patients showed clinical evidence of uremia. Renal failure was confirmed by significant elevation of blood nitrogen.

RESULTS

In Table I are contained the data obtained in thirteen studies on twelve patients. Ten of them were diagnosed clinically as having essential hypertension, one was diagnosed as having acute glomerulonephritis (T. R.), and one, chronic glomerulonephritis (H. R.). The diagnosis of chronic glomerulonephritis was confirmed by necropsy. In addition, five other patients were studied at necropsy. Necropsy showed that one of those (L. L.) with a clinical diagnosis of essential hypertension had chronic glomerulonephritis with an acute exacerbation. One patient (C. S.), diagnosed clinically as having essential hypertension, was found to have a superimposed chronic bilateral pyelonephritis at autopsy in addition to arteriolar nephrosclerosis. The kidney of one patient (A. B.) was studied after nephrectomy done as result of a mistaken clinical diagnosis of unilateral pyelonephritis as the cause of the hypertension. Pathologic examination of this kidney showed a slight amount of pyelonephritis and primarily arteriolar nephrosclerosis. The nephrectomy had no favorable influence on the hypertension. The patient's clinical course was rapidly and steadily downhill and she died in uremia six months after nephrectomy. The studies summarized in Table I on A. B. were done following nephrectomy and after supervention of uremia. Autopsies on L. S., T. D., and W. C. showed arteriolar nephrosclerosis.

Further analysis of the tabulated data shows that high spinal anesthesia produced a marked fall in both systolic and diastolic blood pressure in five patients (S. B., L. S., R. M., C. S., and E. F.). Of the remaining seven patients whose blood pressure fell only moderately (L. L., A. B., E. H., T. D., W. C., H. R., and T. R.) under the influence of high spinal anesthesia, one (T. R.) had acute glomerulonephritis with anuria, two (L. L. and H. R.) had chronic glomerulonephritis (at autopsy), one (A. B.) had proved pyelonephritis in a kidney removed during life, one (E. H.) had an observed clinical course of from four to five years of pyelonephritis, and the remaining two (T. D. and W. C.) had arteriolar nephrosclerosis proved at autopsy.

The initial level from which the blood pressure falls due to high spinal anesthesia is not a determining factor in the extent of the fall, as can be seen by the fact that blood pressure well over 200 mm. Hg was encountered in both individuals with marked falls as well as in those who showed only moderate drops in blood pressure.

Necropsies were done on two (L. S. and C. S.) of the five patients whose blood pressure showed great falls during high spinal anesthesia. The kidneys showed arteriolar nephrosclerosis in both.

DISCUSSION OF RESULTS

Five of twelve patients (S. B., L. S., R. M., C. S., and E. F.) showed extreme falls of blood pressure during high spinal anesthesia. At necropsy two of these (C. S. and L. S.) showed arteriolar nephrosclerosis to be the cause of renal failure. The other three (S. B., R. M., and E. F.) had a clinical diagnosis of essential hypertension with renal failure. Two of the latter three patients (S. B. and E. F.) had clinical manifestations of malignant hypertension. Unfortunately, necropsies were not done on these individuals. These results are in conformity with our original thesis that the blood pressure of patients with sufficiently advanced arteriolar disease to produce uremia should still fall markedly during high spinal anesthesia if the hypertension is of vasomotor origin, and should not fall markedly if the hypertension is of renal humoral origin, any fall being dependent upon improvement in renal blood flow.

Unfortunately, the matter is not as simple as this explanation. This is shown by the fact that seven (L. L., A. B., E. H., T. D., W. C., H. R., and T. R.) of twelve patients failed to show as marked a fall as we obtained in the other five patients and as marked as we have shown in numerous other patients, the study of whom formed the basis of other publications.[1, 2]

In the attempt to explain our failure to obtain marked falls of blood pressure during spinal anesthesia, we analyzed our data further. Three of these seven (L. L., H. R., and T. R.) had acute or chronic glomerulonephritis. The diagnosis of chronic glomerulonephritis was proved by necropsy in two of these (L. L. and H. R.). An additional patient (E. H.) had a justifiable clinical diagnosis of chronic pyelonephritis as shown by our own clinic and hospital records of repeated pyuria, etc., for approximately five years' duration.

We do not understand the significance of the variability in the results obtained in our patients with known nephritis and hypertension. While we realize that it is speculative, it is at least reasonable, on the basis of great accumulation of evidence, to assume that if there is such a thing as hypertension of humoral origin in the human being, it is probably to be found in the individuals with intrinsic renal pathology.[9]

While we have no proof, we assume that the failure to respond with marked drop in blood pressure is explained definitely in three cases (L. L., H. R., and T. R.) and possibly in another case (E. H.) by our further assumption that the pathogenesis of elevated blood pressure is different in essential hypertension and acute and chronic inflammatory disease of the kidney. Unfortunately for this assumption, four patients with proved cases of acute or chronic inflammatory diseases of the kidney (L. L., H. R., T. R., and E. H.) and three with proved cases of arteriolar nephrosclerosis (A. B., T. D., and W. C.) reacted similarly to spinal anesthesia with only moderate drops of blood pressure. In four patients with renal inflammatory disease there was an average fall of 26 mm. Hg systolic and 18 mm. Hg diastolic pressure. In three patients with nephrosclerosis, the average fall was 31 mm. Hg in systolic pressure and 25 mm. Hg in diastolic pressure. While there is a difference, the amount of difference and the few cases do not warrant definite conclusions.

We have not explained failure to obtain a marked fall in blood pressure during spinal anesthesia in three other patients (A. B., T. D., and W. C.), all

of whom had a clinical diagnosis of essential hypertension, two of whom showed at autopsy only arteriolar nephrosclerosis as the cause of renal failure, and one (A. B.) of whom showed, in a surgically removed kidney, arteriolar nephrosclerosis to be the essential lesion plus a slight pyelonephritis.

In the attempt to throw further light on this matter, we have analyzed the data in terms of initial blood pressures. An explanation cannot be found here, as can be seen by the fact that five patients (S. B., L. S., R. M., C. S., and E. F.) had initial systolic blood pressures ranging from 226 to 244. The blood pressures of all these fell markedly during spinal anesthesia. Whereas those patients who did not show much fall during spinal anesthesia (L. L., A. B., E. H., T. D., W. C., H. R., and T. R.) had initial systolic blood pressures of from 178 to 250, five of these (L. L., A. B., T. D., W. C., and H. D.) had initial pressures in the same range as the five who showed such a marked drop.

In view of the acceptance of the idea[10] that renal failure of extreme degree results also in a loss of the metabolic and detoxifying function of the kidney with possible retention of pressor substances, our data have been analyzed from the viewpoint of the difference in the amount of nitrogen retention in patients who showed marked blood pressure falls compared to those who showed slight to moderate falls in blood pressure.

In the five patients who showed extreme falls in blood pressure, the nonprotein nitrogen varied from 75 to 171 mg. per cent, with an average of 114 mg. per cent. In the patients (including those with nephritis—W. C., H. R., and T. R.) who showed only slight to moderate falls in blood pressure, the individual variations in nonprotein nitrogen were from 81 to 300 mg. per cent, with an average of 174 mg. per cent.

At present we are unable to draw any definite or far-sweeping conclusions from the fact that the patients in the group showing the greatest falls in blood pressure had less chemical evidence of uremia than did those in the group which showed only slight to moderate falls in blood pressure. We are, however, postulating the possibility that the blood of patients in uremia contains significant and sufficient amounts of pressor substances, such as aromatic amines or phenols, to explain perhaps the variability of the degree of blood pressure fall during spinal anesthesia. Individual susceptibility to drug action must not be disregarded as a further possibility.

SUMMARY AND CONCLUSIONS

Falls in blood pressure obtained during spinal anesthesia in patients with essential hypertension and uremia may be very marked and as great as falls of blood pressure similarly produced in patients with essential hypertension and normal renal function.

We believe that the great fall in blood pressure induced by high spinal anesthesia indicates that the elevation of blood pressure is due to increased vasomotor tonus even in patients with extreme irreversible arteriolar disease as shown by clinical and chemical evidence of uremia.

These results are contradictory to the thesis that the fall of blood pressure in hypertension produced by functional or anatomical interruption of the sympathetic nervous system is due to improvement in renal blood flow.

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THE EFFECT OF INTRAVENOUS TYPHOID VACCINE ON THE INTRAVENOUS GLUCOSE TOLERANCE TEST

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THE administration of glucose intravenously to replace that usually given orally in carrying out glucose tolerance examinations has been proposed by McKean and associates.[1] The obvious advantages of the test are reduction in the time required (from several hours to fifteen minutes) and obviation of marked discrepancies on repeated examinations caused by irregularities in rate of absorption. The need of a sufficient number of control tests on normal subjects was the immediate incentive for this investigation which was performed on a group of normal soldiers. Later the tests were repeated on a group of soldiers being treated for gonorrheal infections by fever therapy induced by intravenous injections of triple typhoid vaccine. The inclusion of the initial tests from this latter group with those of the normal subjects appeared warranted on the ground that such a local infection would not significantly alter the results.

The tests were made under basal conditions by the intravenous injection of 50 per cent glucose in physiologic salt solution. The dose was equivalent to 0.2 Gm. per kilogram of body weight. The entire volume of sugar solution was injected in exactly one and one-half minutes and glucose determinations were made by the Folin and Wu method on samples of blood taken precisely at three, four, five, ten, and fifteen minutes after the injections were started, without stasis.

These studies were carried out at the Station Hospital, Fort Custer, Mich.
Received for publication, Aug. 23, 1945.

TABLE I. INTRAVENOUS TOLERANCE TESTS

CASE	AGE (YR.)	WEIGHT (POUNDS)	DOSE (GM.)	BLOOD SUGAR (MG. PER CENT)						DATE	DIAGNOSIS	TEMPERATURE (° F.)	PULSE	RESPIRATION
				FASTING	POSTINJECTION									
					3 MIN.	4 MIN.	5 MIN.	10 MIN.	15 MIN.					
1	24	138	12.5	74	154	190	157	153	148	12/17/43	G. C.*	98.6	92	18
2	21	140	12.5	71	125	138	158	118	109	12/14/43	G. C.	99.5	110	23
3	22	173	15.7	72	180	184	156	121	100	12/13/43	G. C.	98.6	82	20
4	22	173	15.7	71	178	173	167	101	94	12/18/43	G. C.	98.0	64	18
5	23	165	15.0	70	160		153	109	102	12/13/43	G. C.	97.6	80	18
6	20	135	12.2	66			160	112	103	12/15/43	G. C.	99.0	88	20
7	37	148	13.2	71	153	163	161	153	141	12/17/43	G. C.	99.0	98	20
8	24	172	15.6	70	178	183	174	151	133	12/16/43	G. C.	98.6	90	18
9	37	171	15.5	76	160	167	174	169	138	12/20/43	G. C.	98.0	80	18
10	23	150	13.6	71	168	160	152	131	116	12/20/43	G. C.	98.2	80	18
11	25	140	12.7	68	179	179	169	142	120	12/15/43	G. C.	99.2	84	20
12	22	165	15.0	70	182	157	150	121	87	12/18/43	G. C.	98.6	86	18
13	21	168	15.0	80	182		178	138	107	12/16/43	G. C.	98.6	74	18
14	19	154	14.0	88	187	196	179	144	129	2/ 7/44	Arthritis			
15	26	160	14.5	87	206	238	235	125	107	8/27/43	Normal			
16	28	180	16.8	87	222.2	229	195.2	190.4	173.0	8/12/43	Normal			
17	47	150	13.6	95	202	208	216	164	154	8/19/43	Normal			
18	26	160	14.5	69	160.0	163.0	153.8	153.8	118	8/12/43	Normal			
19	21	160	14.4	87	129.0	160.0	190.4	133.3	125.0	8/11/43	Normal			
20	27	165	15.0	95	153.8	155.0	166.7	142.8	133	3/10/43	Normal			
21	20	160	14.5	108	142.8	144	144.4	153.8	133	8/13/43	Normal			
22	26	140	12.2	91	181.8	167	160.0	153.0	133	8/10/43	Normal			
23	23	120	11.0	98	154	143	137	148	133	8/ 7/43	Arthritis			
24	22	152	13.5	94	175	192	156	133	128	8/ 7/43	Rheumatic fever			

*Acute gonorrhea.

The results of the tests on the normal control subjects and the preliminary findings in the experimental subjects are given in Table I.

The mean for fasting blood sugar level was 80.4 ± 2.4 mg. per 100 c.c. with a standard deviation of 11.7 ± 1.7. The mean level at the highest points of the curves, which usually occurred at the fourth minute, was 174.6 ± 5.0 mg. per 100 c.c., with a standard deviation of 24.6 ± 3.6. The mean value at the end of 15 minutes was 123.5 ± 4.1 mg., with a standard deviation of 19.9 ± 2.9.

The patients who received the intravenous injections of typhoid vaccine were chosen because the gonorrheal infections had proved resistant to sulfathiazole therapy. The vaccine was the regular triple typhoid used by the Army for inoculation and contained 1,000 million typhoid bacteria and 250 million each of paratyphoid A and B. The amount used for a single injection varied from 25 million to 7,000 million and the number of injections varied from fifteen to twenty for each individual. In each case the amount of vaccine was started at a low level and the concentration gradually increased as it was found that the lower dosages became ineffective. The temperature was raised to from 104 to 106° F. and maintained for several hours. The second glucose tolerance test was taken after the fever had reached the desired temperature. The effect of the typhoid vaccine is shown in Table II. There was a significant increase in temperature from 98.4 to 105.1° F., in pulse rate from 80.0 to 120.2 per minute, and in respiratory rate from 18.6 to 29.5 per minute.

There was no significant difference between the mean values for initial blood sugar level of 71.7 mg. per 100 c.c. when the temperature was normal and that of 78.9 mg. per 100 c.c. when the temperature was elevated. The mean values for the three-minute interval when the blood sugar reached its highest

TABLE II. INTRAVENOUS GLUCOSE TESTS DURING FEVER

CASE	TOTAL MILLION TYPHOID BACTERIA	NUMBER OF DOSES	AMOUNT EACH DOSE	TIME FOR DOSES (HR.)	DURATION FEVER AT TEST (HR.)	BLOOD SUGAR (MG. PER CENT)						TEMPERATURE	PULSE	RESPIRATION
						FASTING	POSTINJECTION 3 MIN.	4 MIN.	5 MIN.	10 MIN.	15 MIN.			
3	825	17	25-100	11	1	74	174	155	147	98	96	104.9	120	24
4*	29,200	16	200-5000	7½	5	91	193	198	182	157	154	105.0	130	25
5	1,300	17	50-200	9	1	76	159	146	138	125	115	104.8	120	35
6	6,500	20	100-400	9½	3½	70	174	167	160	131	118	104.0	120	26
9	15,100	19	50-2000	7	2½	93	177	167	170	165	148	105.4	120	35
10*	15,100	19	50-2000	7	2½	72	143	136	131	119	105	105.4	120	25
11	4,700	18	100-400	8	3	71	163	167	157	138	133	105.0	115	30
12	1,300	16	50-200	9	3	85	154	156	160	154	142	104.8	108	28
12	28,200	15	200-4000	7½	4½	70	169	151	134	126	120	105.0	114	32
13	18,700	19	100-7000	9	4	87	195				145	106.5	135	35

*Developed shock.

value were 173.2 mg. for the control test and 170.1 mg. during the fever. The difference between these values was not significant.

At the fifteen-minute interval the mean blood sugar level was 105.4 mg. for the control test and 127.6 mg. for the test taken during the period of hyperpyrexia. The difference between these readings was 22.2 mg. In eight of the ten subjects, the level taken during the fever was higher than that taken in the control period; in one instance the levels were essentially the same and in the remaining subject the control level was significantly higher. In view of this fact, this difference may be considered significant. The findings are consistent with those of Looney and Borkovic[2] in which the hyperpyrexia was produced by diathermy.

It would appear, therefore, that as a result of fever, no matter what its origin, there is a slowing down in the removal of sugar from the blood. In this regard, one is reminded of the acidosis which frequently occurs in infants and young children during slight fevers and it suggests the possibility that this may be due to a greater susceptibility of the sugar metabolism in these individuals' disarrangement by fever.

The enormous quantities of triple typhoid vaccine which may be administered intravenously is strikingly demonstrated in this series. In half of the subjects, doses of from 2 to 7 c.c. of undiluted vaccine were administered. That the treatment is not without danger was seen by the fact that two of the patients (see Table II) developed shock which in one (Case 10) was so severe as to cause considerable anxiety to the investigators. The shock occurred in these subjects despite the fact that they had on several previous occasions received doses of vaccine as great as they were being given at the time. As a result of this development and because there appeared to be no way of predicting when such a collapse might occur, the investigation was discontinued.

SUMMARY

Glucose tolerance curves were obtained after the intravenous administration of 0.2 Gm. of 50 per cent glucose per kilogram of body weight in twenty-four essentially normal soldiers. The blood sugar level rose from a mean of 80.4 mg. per 100 c.c. to a maximum of 174.6 mg. about the fourth minute and then dropped to 123.5 mg. at the fifteenth minute.

The glucose tolerance curve was elevated after the intravenous administration of triple typhoid vaccine for the treatment of sulfathiazole-resistant

gonorrhea. The mean level at the end of fifteen minutes was 22.2 mg. higher than the initial level of 105.4 mg.

The effect is probably due to the induced fever and not to any specific effect of the vaccine.

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THE DIAGNOSIS OF ENTEROBIASIS

COMPARATIVE STUDY OF THE GRAHAM AND HALL TECHNIQUES IN THE DIAGNOSIS OF ENTEROBIASIS

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WE HAVE previously[1] carried out a comparative study of the techniques of Hall[2] (N.I.H. swab) and of Graham[3] (Scotch cellulose tape loop) for the diagnosis of enteriobiasis. We used both methods, on five successive days, in the examination of seventeen children heavily infected with *Enterobius vermicularis*. Positive results were obtained in 96.5 per cent with Graham's method and in 91.7 per cent with Hall's. In view of the fact that the children in the group tested were heavily parasitized, the comparison was repeated in 240 school children who had not been previously examined. In this second series, 45 per cent positive findings were obtained with Graham's method and 30 per cent with Hall's. Based on these results, we reached the conclusion that the Graham method is the technique to be recommended in the younger age groups, since in adults we considered the Hall technique to be easier to use.

We have subsequently continued to use the Graham method both in children and in adults and have confirmed its superiority by the studies mentioned. It was therefore considered advisable to present the results of these additional studies in 430 individuals, mostly adults, all of whom were examined by both of the techniques under investigation.

MATERIALS AND METHODS

The Graham method was used with the slight modification suggested by us, which consists of using a strip of cellulose adhesive tape 5 cm. in length, without folding the ends, grasping the loop lengthwise with a dissecting forceps. This modification reduces the amount of time required to prepare the loop and the amount of material necessary. The Hall method was carried out exactly as described by Hall.

The examinations were made on the patients of a Surgical Hospital in Mexico City. Each individual was first examined by the Hall technique and then additional material was collected by means of the Graham method.

From the Institute of Health and Tropical Diseases.
Received for publication, Sept. 15, 1945.

TABLE I. RESULTS OBTAINED IN EXAMINATION OF 430 INDIVIDUALS FOR ENTEROBIASIS WITH THE TECHNIQUES OF HALL AND GRAHAM

AGE GROUPS (YR.)	MALES						FEMALES						TOTAL MALES AND FEMALES					
	HALL			GRAHAM			HALL			GRAHAM			HALL			GRAHAM		
	TOTAL NUMBER	NUMBER POSITIVE	PER CENT POSITIVE	TOTAL NUMBER	NUMBER POSITIVE	PER CENT POSITIVE	TOTAL NUMBER	NUMBER POSITIVE	PER CENT POSITIVE	TOTAL NUMBER	NUMBER POSITIVE	PER CENT POSITIVE	TOTAL NUMBER	NUMBER POSITIVE	PER CENT POSITIVE	TOTAL NUMBER	NUMBER POSITIVE	PER CENT POSITIVE
11-20	37	10	27	37	19	51	42	10	24	42	18	43	79	20	25	79	37	47
21-40	75	10	13	75	19	25	118	18	15	118	34	29	193	28	14	193	53	28
41-60	56	8	14	56	16	29	69	8	12	69	18	26	125	16	13	125	34	27
61-80	17	3	18	17	6	35	16	1	6	16	6	38	33	4	12	33	12	36
Totals	185	31	17	185	60	32	245	37	15	245	76	31	430	68	16	430	136	32

RESULTS

As can be seen from Table I, the Graham method proved to be much more efficient than the Hall technique. The total percentage of positive findings was 32 per cent with the Graham method and 16 per cent with the Hall method. In all age groups, without regard to sex, the percentage of positive results obtained with the Graham method was significantly higher.

DISCUSSION

The findings presented confirm the superiority of the Graham method, even in adults with perianal hairs, which includes almost all the adult males. The considerable percentage of positive findings obtained justifies the use of this procedure also in adults, since the minimal discomfort which may be occasioned by its use is well tolerated by all patients.

As indicated previously, in each case, material for the Hall method was collected first so that actually it enjoyed a small advantage over the Graham method.

SUMMARY

Examinations for the diagnosis of enterobiasis were carried out in 430 individuals using both the Hall and Graham techniques. The percentage of positive results obtained was 32 per cent with the Graham method and 16 per cent with the technique of Hall.

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THE URINARY EXCRETION OF ANTIPERNICIOUS ANEMIA FACTOR

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A NUMBER of investigators have suggested that the antipernicious anemia factor (A.P.A.) may be excreted in the urine of human subjects;[1, 2, 13] the evidence offered in support of this possibility, however, has not been conclusive. Since the A.P.A. factor* may be a relatively small molecule,[3] one would not be surprised to find that it passed readily through the glomerular filter, so that after the injection of a large amount of liver extract, a portion of the active principle might be found in the urine. However, the administration of 300 U.S.P. units of liver extract in one intramuscular injection to a patient with pernicious anemia in relapse has been observed to induce a remission which lasted for nine months,[4] and Seymour, Heinle, and Miller[5] found that when

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Aided in part by the Nathan Greenberg Research Fund.

Received for publication, Oct. 3, 1945.

*That the liver principle may be multiple in nature, as claimed by Jacobson and Subbarow,[3] is realized. For the sake of convenience, however, it will be referred to as A.P.A. factor rather than factors.

patients in remission were given from 60 to 140 U.S.P. units of liver extract over a short period of time, relapse did not occur for from nine to thirty weeks. These observations suggest that the urinary loss of the injected active principle could not have been great, but they must be interpreted with caution because Goldhamer[6] has shown that the deficiency of intrinsic factor in gastric secretion is relative rather than absolute. The amount of supplementary A.P.A. factor required daily by patients with pernicious anemia, therefore, probably varies with the degree of deficiency of the intrinsic factor. This study was undertaken to determine more definitely how much of the A.P.A. factor is excreted promptly into urine after parenteral administration of liver extract.

In previous studies it has been shown[7-12] that a substance is present in the urine of normal individuals and of patients with pernicious anemia which is capable of stimulating the production of reticulocytes in pigeons, rats, and guinea pigs. However, since this reticulocytogenic substance is not known to be identical with, or related to, the A.P.A. factor, investigations using these test animals have not provided conclusive evidence that the A.P.A. factor is found in human urine.

A more satisfactory approach to the problem has been made by investigators who have used patients with pernicious anemia in relapse for assaying urine extracts. Decastello[2] reported in 1935 that urine from normal subjects and from patients with pernicious anemia, when given rectally or intramuscularly, was effective in the treatment of pernicious anemia. However, his results were not uniform, and Jequier and Apsey[13] were not able to confirm them. Wakerlin,[14] after reporting that a patient with pernicious anemia did respond to intramuscular injection of a urine extract, was unable to duplicate his results.[15] Thornley[16] was not able to obtain A.P.A. activity from 1,750 c.c. of normal human urine with a method of extraction which had consistently yielded potent antianemia fractions from liver; his assay was done on a patient with pernicious anemia in relapse. In 1940, Wilkinson[1] found that extracts made from 20 to 40 liters of urine obtained either from normal individuals or from patients with pernicious anemia in remission induced hematologic responses in several patients with pernicious anemia in relapse; similar extracts prepared from the urine of untreated patients with pernicious anemia had no therapeutic value. He used two methods of extraction: (1) The urine was concentrated directly, taken up in 90 per cent alcohol, reconcentrated, precipitated in absolute alcohol, and dried; (2) the urine was treated with Norite, eluted from the Norite by 60 per cent alcohol, concentrated, precipitated in absolute alcohol, and dried.

Because Wilkinson's observations provide the strongest evidence so far accumulated that the principle in liver effective in the treatment of pernicious anemia is excreted in urine, his data are reviewed in more detail as follows: (1) Extracts made from the urine of normal individuals were injected intramuscularly in doses equivalent to from 8.5 to 20 liters into three patients with pernicious anemia in relapse. Reticulocyte responses of 14.8, 11.6, and 12.5, respectively, were stimulated, and a rise in the red cell count of 0.77 and 0.62 million per cubic millimeter occurred in two patients; erythrocyte values were not reported for the third patient. (2) Extracts made from the urine of patients with pernicious anemia in remission and given intramuscularly in doses equivalent to from 10 to 22.6 liters to three patients with pernicious anemia in relapse caused reticulocyte responses of 12.2, 29.6, and 15.2 per cent and rises in the erythrocyte level of 0.82, 1.34, and 1.44 millions per cubic millimeter.

(3) Extracts made from the urine of patients with pernicious anemia in relapse failed to induce a reticulocyte response. It is emphasized that Wilkinson used large amounts of urine for his extracts and made no attempt to determine whether the A.P.A. factor from injected liver extracts was excreted in significant amounts.

In the study which forms the basis for this report, it was determined that urine excreted by subjects during the twenty-four hours following a relatively large injection of liver extract contained no detectable A.P.A. activity. Control observations demonstrated that the method used for extracting urine was satisfactory; A.P.A. factor which had been added to urine directly could be recovered.

METHODS

The simplest possible method of extraction was used in order that loss of activity in the extraction process might be minimized. Urine was evaporated to near dryness under reduced pressure at 46° C. Then 95 c.c. of 70 per cent ethyl alcohol were added and the mixture filtered; the filtrate was evaporated nearly to dryness under reduced pressure at a temperature not greater than 50° C. The residue was taken up in sterile water to give a final volume of from 27 to 40 c.c., and phenol was added as preservative to give a final concentration of 0.5 per cent.* The solid content of each preparation was determined. Each extract was cultured aerobically and anaerobically and used only after it had been found to be sterile. Subcutaneous injection of 1 c.c. into a dog produced only slight induration after twenty-four hours. All of the extracts caused pain at the site of intramuscular injection. In order to lessen the discomfort, the daily dose was diluted with water (from 2 to 8 c.c.). One cubic centimeter of 1 per cent novocain was added to the syringes for administration of Extracts 2 and 4.

Four extracts were prepared:

Extract 1.—Ten cubic centimeters of a commercial liver extract† containing 100 U.S.P. units of A.P.A. factor were added to 1 liter of normal human urine before it was evaporated. Solid content of the extract equalled 36.5 per cent and final volume was 27 c.c.

Extract 2.—Ten cubic centimeters of a commercial liver extract‡ containing 100 U.S.P. units of A.P.A. factor were added to 1 liter of normal human urine before it was evaporated. Solid content of the extract equalled 55.9 per cent and final volume was 30 c.c.

Extract 3.—A healthy adult male (R. H. R.) was injected intramuscularly on each of three successive days with 10 c.c. of a liver extract‡ containing 100 U.S.P. units of A.P.A. factor. Urine was collected for a twenty-four hour period beginning with the last injection. This urine (1,175 c.c.) was extracted. Solid content was 59 per cent and final volume was 40 c.c.

Extract 4.—A patient with pernicious anemia in relapse (E. G.), who had been given a daily injection of 1 U.S.P. unit of A.P.A. factor for fifteen days, was on each of five subsequent days given intramuscularly 5 c.c. of Reticulogen† containing approximately 100 U.S.P. units per 5 c.c. On the fifth day, a twenty-four hour clean-voided urine specimen (1,790 c.c.) was collected and extracted. Solid content equalled 51.8 per cent and final volume was 30 c.c.

Red blood cell counts, hemoglobin levels, and reticulocyte percentages were determined daily on the capillary blood of the test subjects. Trenner pipettes and counting chambers were standardized by the United States Bureau of Standards. The hemoglobin determinations were made by the oxyhemoglobin method of Evelyn.[17] Both wet and dry films were stained with brilliant cresyl blue for counting reticulocytes.

*Initial attempts to recover the A.P.A. factor from urine by absorbing it on Darco g60 and eluting the Darco with ammoniacal alcohol were unsuccessful.

†Eli Lilly and Company, Indianapolis, Ind.

‡Wilson Laboratories, Division of Wilson & Co., Inc., Chicago, Ill.

Test subjects were patients with pernicious anemia in relapse who were shown to have the peripheral blood and bone marrow changes characteristic of the disease and a histamine refractory achlorhydria. They were given a meat-free diet and were observed for a control period of at least five days before assays were begun.

RESULTS

1. *Proof That Added A.P.A. Factor Can Be Recovered From Urine.*—The first two extracts were made from urine to which liver extract had been added directly. They were assayed to prove that the extraction method permitted the recovery of A.P.A. factor. Daily intramuscular injections of 0.5 c.c. of Extract

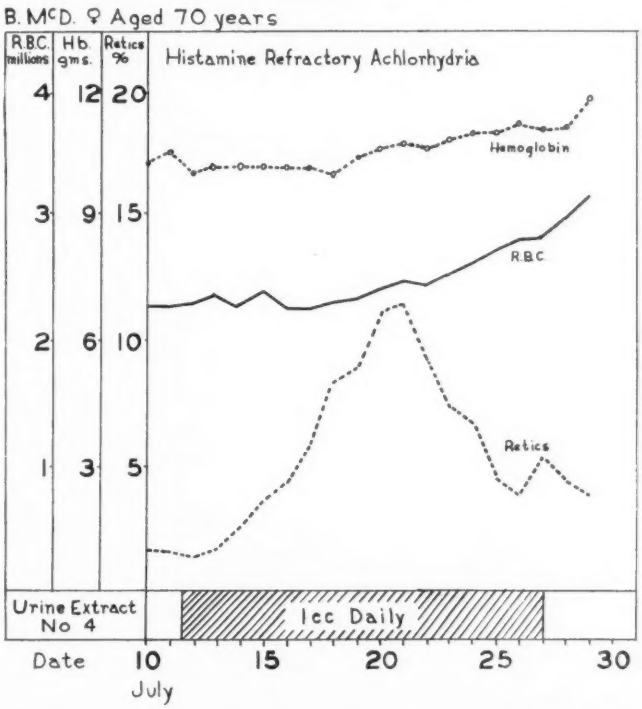

Fig. 1.—Hematologic response induced in a patient with pernicious anemia by an extract made from urine to which liver extract had been added.

1 diluted with 4.5 c.c. of sterile water were given for eight and nine days, respectively, to two test subjects (Table I, R. M. and K. N.). In the first of these (R. M.), a submaximal reticulocyte response of 17.2 per cent developed on the eleventh day. The occurrence of cystitis with accompanying fever (as high as 39.4° C.) during the assay period may well have prevented the reticulocytosis in this patient from reaching the level of 24 per cent, which would have been expected[18] had the therapeutic effect been maximal. The second subject showed the maximum expected reticulocyte rise and an increase in the erythrocyte level of a million cells. In all probability, therefore, each 0.5 c.c. of this extract contained at least 1 U.S.P. unit of the A.P.A. factor. Since the total volume of the extract was 27 c.c., at least 54 U.S.P. units of the original 100 units added to the urine were recovered.

Extract 2 was given intramuscularly in daily doses of 1.0 c.c. (diluted with 2 c.c. of sterile water and 1 c.c. of 1 per cent novocain) for seventeen days to a third test patient (Table I and Fig. 1, B. McD.). A reticulocytosis of 11.4

TABLE I. ASSAYS OF URINE EXTRACTS FOR ANTIPERNICIOUS ANEMIA FACTOR MADE ON PATIENTS WITH PERNICIOUS ANEMIA IN RELAPSE

	SUBJECTS USED FOR ASSAY			TYPE OF EXTRACT DAILY DOSE (INTRAMUSCULAR)	HEMATOLOGIC DATA			
	PATIENT	SEX	AGE (YR.)		DAY AFTER START OF THERAPY	R.B.C. (MILLIONS)	HB. (GM.)	RETICULOCYTES (%)
1. Assay of urine extracts prepared after 100 U.S.P. units of liver extract had been added to 1 liter of urine	R. M.	F	62	0.5 c.c. Extract 1 for 8 days*	0	1.75	8.9	2.0
					6	1.64	8.3	3.9
					11	1.95	8.8	17.2
				50 U.S.P. units of liver extract for 6 days†	0	1.95	8.8	17.2
					4	1.97	9.0	14.1
					6	2.25	9.8	24.3
					13	2.82	11.9	9.0
	K. N.	F	61	0.5 c.c. Extract 1 for 9 days	0	1.80	8.2	0.8
					4	1.41	7.6	3.4
					9	2.00	8.7	27.1
					16	2.35	10.1	11.0
					19	2.72	11.2	5.3
	B. McD.	F	70	1.0 c.c. Extract 2 for 17 days	0	2.31	10.5	1.7
					5	2.41	10.2	3.8
					11	2.48	10.8	11.4
					19	3.16	12.0	4.0
2. Assay of extracts prepared from urine collected for twenty-four hours after liver extract had been given parenterally	E. G.	M	47	2.0 c.c. Extract 3 for 9 days	0	1.39	5.1	1.2
					3	1.47	5.3	2.0
					6	1.39	5.5	1.6
					9	1.30	5.3	1.2
				1 U.S.P. unit of liver extract‡ for 15 days; then 100 U.S.P. units each day for the following 5 days	0	1.30	5.3	1.6
					6	1.30	5.5	8.2
					12	1.50	6.3	26.5
					26	2.49	9.5	10.0
					46	3.84	11.3	1.6
					116	4.84	13.4	----
	F. W.			1.0 c.c. Extract 4 for 11 days	0	2.60	10.1	4.2
					4	2.60	10.3	4.5
					8	2.66	10.1	3.0
					11	2.62	9.6	4.0
				2 U.S.P. units of liver extract for 12 days‡	0	2.64	9.8	3.3
					4	2.64	10.2	5.4
					8	2.86	10.7	19.4
					12	3.19	12.3	10.5
					20	3.70	14.9	3.8
					41	4.58	14.7	----

*Patient developed urinary tract infection on second day of therapy; treated with sulfadiazine.
†Commercial liver extract made by the Lederle Laboratories, Inc., Pearl River, N. Y.; 10 U.S.P. units per cubic centimeter.
‡Commercial liver extract made by the Wilson Laboratories, Division of Wilson & Co., Inc., Chicago, Ill.; 10 U.S.P. units per cubic centimeter.

per cent occurred on the eleventh day and the erythrocytes increased from 2.3 to 3.16 million cells per cubic millimeter. This response may also be interpreted as indicating that at least 1 U.S.P. unit of active principle was present per cubic centimeter. Since the total volume of the extract was 30 c.c., one can only conclude that at least 30 of the 100 units added to the urine before extraction were recovered. No attempt was made to refine the assay further in an attempt to make a quantitative estimate of the recovery.

2. *Absence of Detectable A.P.A. Activity in Extracts Prepared From Urine of Subjects Who Had Received Liver Extract Parenterally.*—Extracts 3 and 4 were prepared from urine excreted by two subjects during the twenty-four hours immediately following the last of three and five daily injections of 100 U.S.P. units of liver extract. One of these subjects was a healthy man. The second was a patient with pernicious anemia. Selection of this latter patient was made in order to eliminate the possibility that persons with this disease

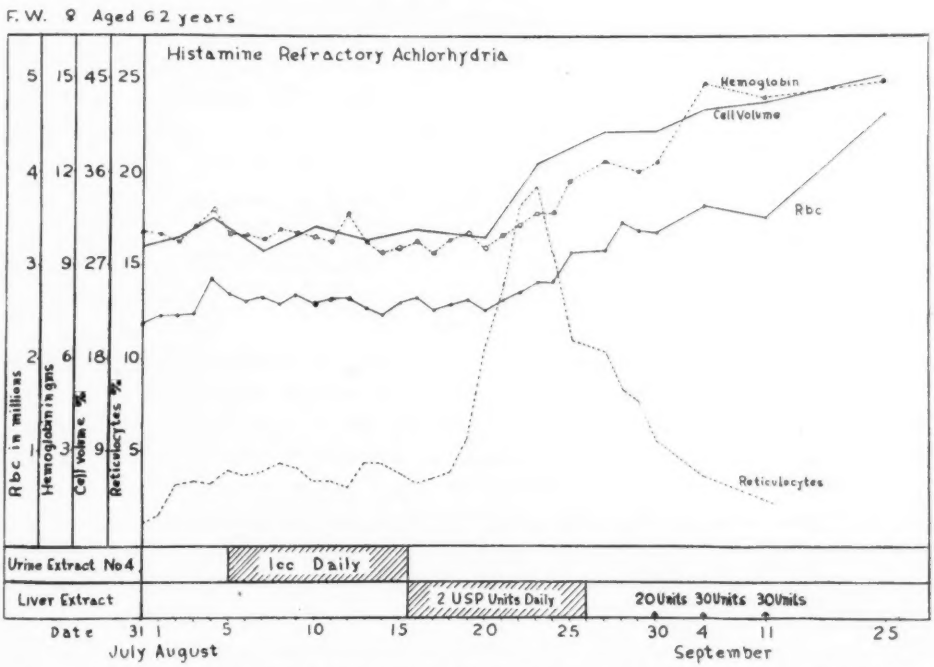

Fig. 2.—Absence of detectable A.P.A. activity in extract prepared from urine of subject who had received liver extract parenterally.

might behave differently from normal individuals in their ability to excrete A.P.A. factor. Neither extract stimulated a reticulocyte response. Two cubic centimeters of Extract 3, diluted with 8 c.c. of sterile water, were injected intramuscularly each day for nine days into a patient with untreated pernicious anemia (Table I, E. G.). No significant change occurred in either the reticulocyte or red blood cell levels. From the tenth to the twenty-fifth day, 1 U.S.P. unit of a commercial liver extract* was administered intramuscularly each day; the reticulocytes rose to 25 per cent. The total volume of Extract 3 was 40 c.c. Each daily dose, therefore, corresponded to 5 per cent of the total volume. If as few as 20 U.S.P. units had been excreted, and the recovery had been only 50 per cent, some reticulocyte rise should have occurred. In the absence of any effect, it seems reasonable to conclude that less than this amount was excreted by the kidneys.

*Wilson Laboratories, Division of Wilson & Co., Inc., Chicago, Ill.

*Commercial liver extract made by the Wilson Laboratories, Division of Wilson & Co., Inc., Chicago, Ill.; 10 U.S.P. units per cubic centimeter.

Extract 4 in daily doses of 1 c.c. failed to produce a hematologic response in one test subject (Table I and Fig. 2, F. W.). This test patient, however, did respond characteristically when she received 2 U.S.P. units of liver extract per day. The subject from whom urine was collected for preparation of this extract was a man with pernicious anemia who had served as a test patient for assay of Extract 3. After the diagnosis and his ability to respond normally to administration of liver extract had been established, he was given 100 U.S.P. units of liver extract on each of five days. Urine was collected during the twenty-four hour period which immediately followed the last injection.

These observations served to establish two things: (1) With the methods used, no detectable A.P.A. activity could be extracted from urine excreted within twenty-four hours after a relatively large dose of liver extract; (2) the data rule out the possibility that the reticulocyte responses obtained with Extracts 1 and 2 were nonspecific.

DISCUSSION

If the A.P.A. factor is a relatively small molecule, it is rather surprising that after large doses were given parenterally detectable amounts failed to appear in the urine. The result, however, is compatible with the clinical experience summarized in the introductory paragraph which indicates that a great proportion of the active principle is stored even when it is given in large amounts. Nevertheless, from Wilkinson's observations[1] that small amounts of A.P.A. substance can be recovered from large volumes of urine, one would logically expect that after parenteral administration of liver extract there might temporarily be an increased urinary output of A.P.A. factor.

In the clinical management of patients with pernicious anemia, however, there are many reasons for being cautious about recommending that the interval between injections be lengthened to more than six or eight weeks; for instance, the desirability of observing the patients at regular intervals, the fear that the antianemia factor may not be identical with the substance required for the control and prevention of combined system disease, the possibility that some intercurrent disease might increase the requirement for A.P.A. factor so as to disrupt calculations as to requirement, and the probability that sensitization to liver extract occurs more frequently as intervals between injections are prolonged. The results of the present study would suggest that large amounts of liver extract injected at widely spaced intervals might be stored efficiently.

SUMMARY

1. A method has been described by which at least 50 per cent of the A.P.A. factor can be extracted from urine containing a known amount of this substance.

2. No A.P.A. activity could be detected in the urine excreted by two subjects during the twenty-four hours immediately following the last of from three to five daily injections of 100 U.S.P. units of liver extract.

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THE ACTIVITY OF SYNTHETIC LACTOBACILLUS CASEI FACTOR ("FOLIC ACID") AS AN ANTIPERNICIOUS ANEMIA SUBSTANCE

I. Observations on Four Patients: Two With Addisonian Pernicious Anemia, One With Nontropical Sprue and One With Pernicious Anemia of Pregnancy

Carl V. Moore, M.D., Olga S. Bierbaum, B.S., Arnold D. Welch, Ph.D., M.D., and Lemuel D. Wright, Ph.D.

ADMINISTRATION of the synthetic *Lactobacillus casei* factor of Angier and co-workers[1] stimulates a reticulocyte response and an increase in the red blood cell count in patients with macrocytic anemia in relapse.[2a, b, c] Spies and associates[2a] observed these hematologic effects in nine patients, four of whom were given the *L. casei* factor by mouth and five by parenteral routes. The oral dose was either 100 or 150 mg. daily; one patient was given a single intravenous injection of 20 mg., another was given 50 mg., while three patients were given daily injections of 20 mg. each. These patients are described only as having "macrocytic anemia in relapse." Data were not reported which would make it possible to determine whether they had nutritional macrocytic anemia or Addisonian pernicious anemia. In patients seen at the Nutrition Clinic of the Hillman Hospital, it frequently is difficult to differentiate, without long periods of study, between these two types of macrocytic anemia.[3] Darby and Jones[2b] obtained satisfactory remissions in two patients with sprue who were given 15 mg. of the same material intramuscularly per day. More recently Spies and co-workers have noted similar effects in several patients with tropical sprue.[2c] The findings described in the present communication confirm and extend these observations. Satisfactory hematologic and clinical remissions have been induced by the administration of synthetic *L. casei* factor to two patients with Addisonian pernicious anemia, to one patient with nontropical sprue, and to one patient with pernicious anemia of pregnancy. Data are presented which indicate that the compound has antipernicious anemia rather than extrinsic factor activity.

Before these results are presented, however, use of the term "*Lactobacillus casei* factor" instead of "folic acid" should be explained. "Folic acid" is the name given by Mitchell, Snell, and Williams[4] to a substance, obtained in nearly pure form from spinach, which supports growth of two organisms commonly used in microbiologic investigation: *L. casei* and *Streptococcus lactis R* (now termed *S. faecalis*). Its potency for *S. lactis R* was used as a guide in purification procedures, and "folic acid" is defined specifically as a growth factor for that organism.[5] A number of other substances with similar physiologic properties have more recently been isolated from liver, yeast, or other sources; at least three of these substances have been obtained in crystalline form. Some of these compounds either will not support the growth of *S. lactis R*, or affect its

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The expenses for this study were defrayed in part by the Phillip Hunkel Research Fund.

The synthetic *L. casei* factor used in these studies was supplied by the Lederle Laboratories, Inc., Pearl River, N. Y., through the courtesy of Dr. Benjamin Carey.

Received for publication, Dec. 3, 1945.

growth less markedly than that of *L. casei*, and it is, strictly speaking, incorrect to designate them as "folic acids." "*L. casei* factor (s)" is a more appropriate, but less euphonious, generic term.[6]

More specifically, the synthetic material supplied to us is referred to as *L. casei* factor because it is reported to be identical with a crystalline preparation isolated from liver by Stokstad.[7a] This substance, which was termed "liver *L. casei* factor," was equally potent as a growth factor for the two bacteria mentioned and appeared to be the same as crystalline vitamin B_c extracted from liver by Pfiffner and co-workers.[8] On the basis of data from absorption spectra, Hutchings and his associates concluded that it could not be identical with "folic acid" prepared from spinach.[7b] The terminology is confusing and the relation of these several factors to each other is not entirely clear. For a more complete statement of the problem, reference should be made to the recent review by Daft and Sebrell.[6] The chemical structure of the "liver *L. casei* factor" has not been announced, although its synthesis has been reported by Angier and associates.[1]

It is not surprising that the synthetic *L. casei* factor has been found to have antianemic properties in man; experiments have abundantly demonstrated that identical or closely related nutrilites influence the metabolism of bone marrow in experimental animals. For instance, crystalline vitamin B_c prevents[9] the Hogan-Parrott type[10] of nutritional anemia in chicks. Complete remissions in three monkeys with nutritional cytopenia (formerly termed vitamin M deficiency and characterized by anemia, leucopenia, and thrombocytopenia) treated with a highly purified preparation of *L. casei* factor* are described in a recent report.[11] Rats fed a highly purified diet containing all the other known components of the vitamin B complex and one of the sulfonamide drugs develop hypocellularity of the bone marrow, leucopenia, granulocytopenia, and an anemia.[12a] All of these manifestations are corrected by liver extracts known to contain *L. casei* factor, by crystalline vitamin B_c, and by a crystalline *L. casei* factor.*[12b]

Although consideration was given by Wright and Welch[12c] to a possible relationship of "folic acid" to pernicious anemia, earlier attempts to discover therapeutic effectiveness of "folic acid" or of *L. casei* factor for anemias or leucopenias in man yielded indifferent results. The present authors assayed several concentrates of "folic acid" in 1943 for extrinsic factor (Castle) activity. These concentrates were prepared either from grass juice** or from spinach. Patients on whom the assays were done had classical hematologic and clinical manifestations of pernicious anemia, and were fed a meat-free diet. To the first subject, 500 mg. of a crude concentrate*** from grass juice were given each day for fifteen days together with 125 c.c. of normal human gastric juice. Reticulocytes began to increase on the fifth day of this period and reached a maximum of 25.6 per cent on the fourteenth day; meanwhile the red blood cell count rose from 1.3 to 1.7 million cells per cubic millimeter. That the response had been submaximal was indicated by the fact that an additional small reticulocyte rise occurred when 1 oral U.S.P. unit of liver extract was given daily beginning with the sixteenth day. This result seeemd to indicate that such concentrates might contain some extrinsic factor activity. Although several other patients were given concentrates without comparable responses, some of these patients were found subsequently to respond very poorly to oral liver extracts. Among the preparations studied was a more highly purified sample of "folic acid," prepared from spinach and kindly presented to us by Dr. R. J. Williams of the University of Texas. Fifteen milligrams of this material given daily for eleven days, together with 125 c.c. of normal human

gastric juice, supplied an amount of "folic acid" equivalent to that contained in the cruder concentrate given previously. In this dosage, the preparation failed to produce a significant rise in reticulocytes or erythrocytes (the red cell count initially was about 1.4 million per cubic millimeter). When the patient was given 1 U.S.P. oral unit of liver extract daily, the reticulocytes, by the fifteenth day, had risen to 18.5 per cent and the erythrocyte count was 2.37 million per cubic millimeter.

This patient had previously been given for ten days, without any significant response, daily intramuscular doses of 5 c.c. of a crude preparation of *L. casei* factor, containing in each dose from 0.06 to 0.12 mg. of microbiologically active material.

Negative results were obtained by Castle and his associates[13] when vitamin-free casein and all the crystalline members of the vitamin B complex including "folic acid and folic acid concentrate" in amounts of 2.3 to 3.6 mg. daily were added to normal gastric juice and fed daily to patients with pernicious anemia in relapse.

Sharp, Vonder Heide, and Wolter[14] gave daily doses of a yeast concentrate said to contain 0.6 to 1.5 mg. of vitamin B_c to ten patients with anemias that had proved refractory to liver and iron. They obtained "an appreciable increase in the hematocrit but only slight changes in other erythropoietic phenomena." Watson and his associates[15] gave 5 mg. of *L. casei* factor[7b] daily by mouth for six days to eight patients with refractory anemia, and one patient with leucopenia persisting after sulfonamide therapy; no therapeutic effect was obtained in any case. Elevations of the leucocyte count, however, were produced by similar doses administered to 7 patients who had developed leucopenia following roentgen-ray therapy; the elevations in the white counts did not persist after the *L. casei* factor was discontinued.

METHODS AND MATERIAL

The hematologic techniques used in this investigation have been described in an earlier publication.[3] Fat determinations on stool specimens were performed according to the method described by Hanes.[16]

All patients in the study were hospitalized and all, with the exception of the woman with nontropical sprue, were fed a meat-free diet. Observations were made during control periods of two to five days or more and complete blood counts were made daily. Changes in the peripheral blood and bone marrow in each instance were those characteristic of pernicious anemia in relapse. Results of gastric analyses and fluoroscopic studies of the gastrointestinal tract are recorded in case summaries at the end of this paper. Two healthy laboratory workers served as control subjects; one took 100 mg. of synthetic *L. casei* factor orally each day for 10 days, the other was given daily intravenous injections of 20 mg. for a similar period.

When the synthetic compound was given orally, it either was placed in capsules or was suspended in water. For parenteral administration, 100 mg. of synthetic *L. casei* factor and 100 mg. of sodium bicarbonate were added to 20 c.c. of freshly distilled water, placed in a serum vial, and autoclaved for 15 minutes at 15 pounds of pressure. The solution was then clear. New solutions were prepared at least once every three days. Sterilization through a Seitz filter was abandoned because of uncertainty concerning the amount of absorption of the vitamin by the filter. All weighings were accurately made on an analytical balance. Injections were given rapidly and no reactions were observed.

RESULTS

1. *Oral Administration of Synthetic L. casei Factor ("Folic Acid") to Two Patients With Addisonian Pernicious Anemia and to One Normal Control Subject.*—The synthetic *L. casei* factor was given orally to two patients with true pernicious anemia because it was felt that a therapeutic effect would not be induced if the material behaved as Castle's extrinsic factor, unless normal human gastric juice were administered at the same time. On the other hand, if the material stimulated a remission without added gastric juice, the result would constitute presumptive evidence that the compound possessed antipernicious anemia factor activity. The first patient treated (M.G.) was given 100 mg. of the synthetic preparation orally each day for ten days. Her initial red blood cell level was approximately 1.2 million cells per cubic millimeter. On the third day of therapy she experienced the sense of well-being and the increased appetite which usually occurs with liver therapy at this time. On the following day her reticulocytes began to rise and a peak value of 40 per cent was reached on the seventh day (Fig. 1). Her red blood cells began to increase in number on about the seventh day and the count rose rapidly until a level slightly over 3.0 million cells was attained. At this point the rise stopped (therapy was discontinued after the tenth day) and a plateau has existed for nearly two weeks. Further therapy was intentionally withheld in order that we might determine how high the erythrocyte level would go following ten days of dosage (1 gram).

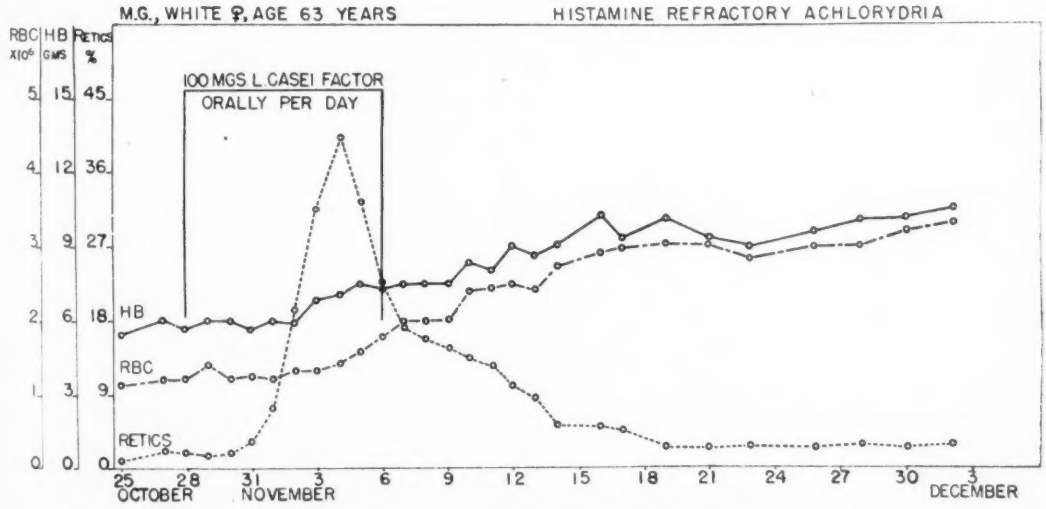

Fig. 1.—Hematologic response of a patient with Addisonian pernicious anemia to the oral daily administration of 100 mg. *L. casei* factor.

To the second patient (E.K.), a daily oral dose of 30 mg. was given for fourteen days. This woman was 78 years of age and had a red blood cell count of only 0.7 to 0.95 million cells per cubic millimeter at the time of her admission to the St. Louis City Hospital. Because of her critical condition, a transfusion of 500 c.c. of citrated whole blood was given. This increased her erythrocyte count to 1.4-1.5 million cells per cubic millimeter. The reticulocytes began to increase on the fourth day of therapy; a maximum of 44.5 per cent occurred on the eighth day (Fig. 2). The rise in the red cell count began on the sixth day and was as rapid as is usual with liver therapy. This response is particularly noteworthy because the patient developed bronchopneumonia and a

decubitus ulcer on the sixth day; she was given penicillin and was digitalized with Digoxin injected intravenously. She was critically ill for three days but is now greatly improved. The irregularity of the erythrocyte curve from the sixth to the tenth days is probably attributable in part at least to the degree of dehydration present during that period. On the fourteenth day, her red blood cell count was 2.5 million per cubic millimeter. Attention is also directed to the fact that the original leucopenia and thrombocytopenia were corrected by the *L. casei* factor. During the period of pneumonia, there occurred a leucocytosis of 22,800 cells per cubic millimeter.

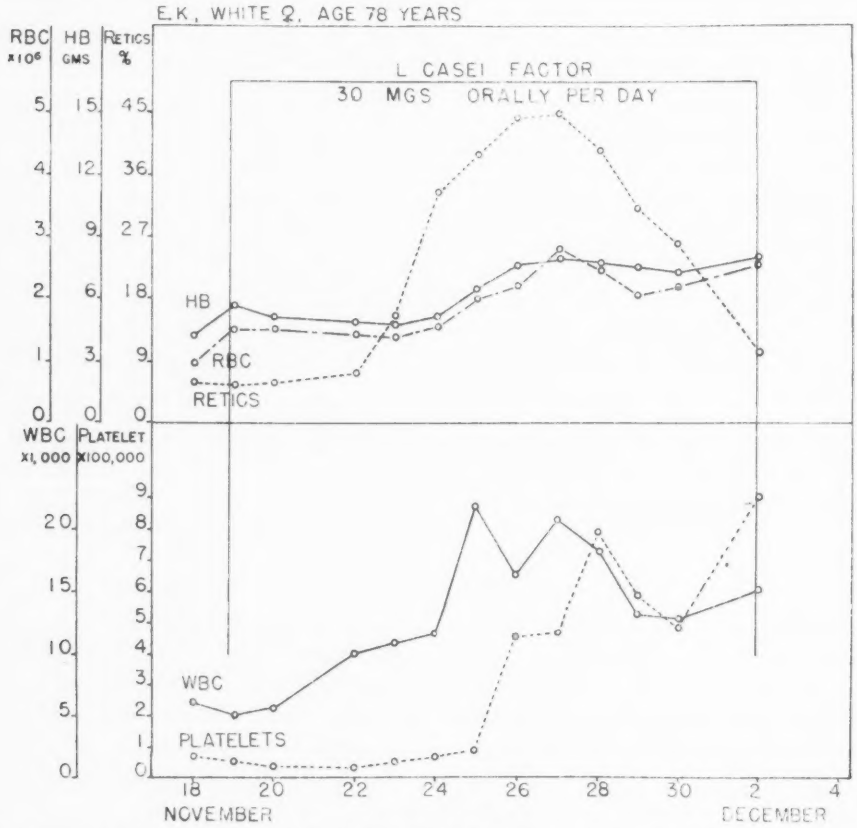

Fig. 2.—Hematologic response of a patient with Addisonian pernicious anemia to the daily oral administration of 30 mg. *L. casei* factor.

The normal control subject, who ingested 100 mg. of the synthetic *L. casei* factor daily for ten days, showed no change in reticulocytes, red cells, hemoglobin, leucocytes, or platelets during three weeks of observation. She noted no symptoms and no subjective changes.

2. *Intravenous Administration of Synthetic L. Casei Factor ("Folic Acid") to One Patient With Nontropical Sprue, to One Patient With Pernicious Anemia of Pregnancy, and to One Normal Control Subject.*—Twenty milligrams of synthetic *L. casei* factor were injected intravenously each day for ten days to J.S., a woman 54 years of age who had all the classical manifestations of nontropical sprue. The oral glucose tolerance curve was flat while the intravenous tolerance test was normal, stool fat varied from 50 to 75 per cent of dry weight, the serum calcium was low while alkaline phosphatase was high, and weight loss had been extreme. Following the initial ten-day period, 40 mg. were injected

every other day for an additional two weeks. The number of reticulocytes began to increase on the third day of therapy and a value of 30.2 per cent was attained on the seventh day, in spite of the fact that her initial red blood cell count was 2.6 million cells (Fig. 3). The authors have never seen a reticulocytosis of this degree following the parenteral administration of liver extract to patients with pernicious anemia who had comparable red blood cell counts. Subjective clinical improvement occurred, but this change was not dramatic; the amount of fat in the stool did not decrease until after the fat in her diet had been decreased from about 200 to 70 grams per day. The erythrocyte count rose to approximately 3.5 million cells, at which level the mean corpuscular volume had become normal (88 cubic microns) and the mean corpuscular hemoglobin concentration had decreased to 28 per cent. It is of interest that this patient had a hypochromic microcytic anemia four months previous to the present hospitalization (see case summary). Leucocytes began their climb to normal levels on the third day; platelets began to increase on the sixth day. Values for both these formed elements are now normal one month after institution of therapy. The patient has gained 6 pounds in weight within four weeks; her oral glucose tolerance test still shows poor absorption.

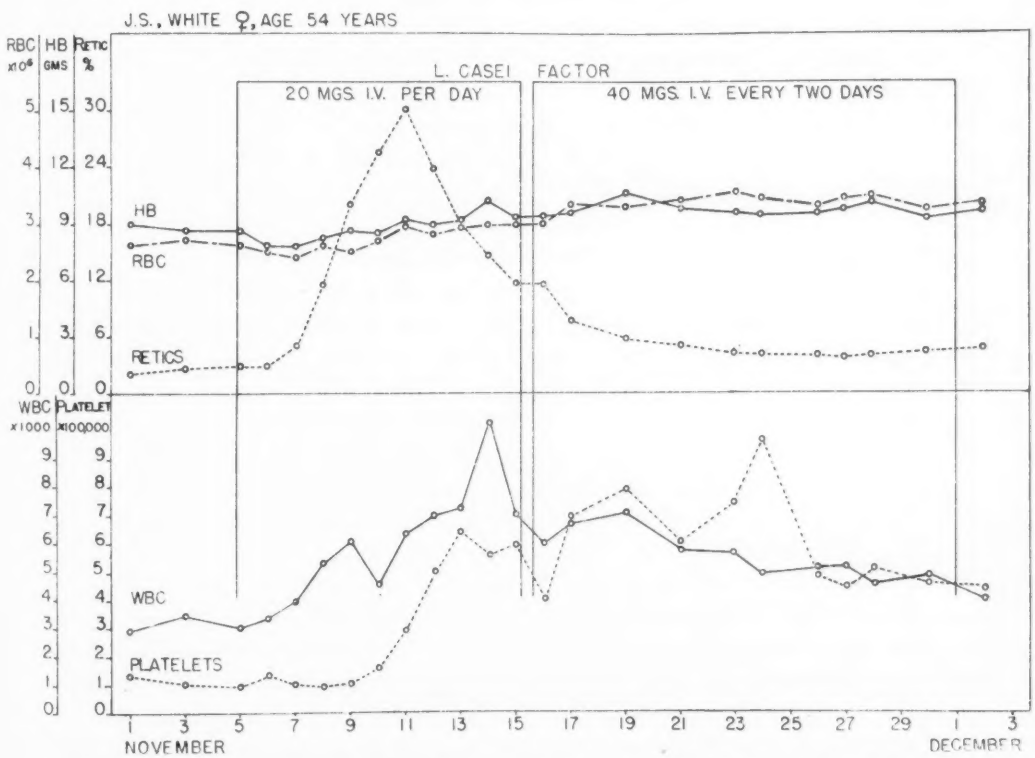

Fig. 3.—Hematologic response produced in a patient with a macrocytic anemia of nontropical sprue by the daily intravenous administration of 20 mg. *L. casei* factor.

The patient with pernicious anemia of pregnancy (M. W.) is a young woman 32 years of age who had developed a similar anemia during each of four previous pregnancies. Liver therapy was given when her third and fourth children were born but she was not carefully followed; she remained subjectively well between pregnancies. She ate liver ordinarily about twice a month but took no additional amounts. At the time of parturition on Oct. 24, 1945, in another hospital, she was found to have a red blood cell count of only 1 million cells. Transfu-

sion was refused; liver extract was not given; and she signed out of the hospital under protest. Because she was totally unable to do her work and was very weak, she asked to be admitted to the St. Louis City Hospital on Nov. 11, 1945. Her red blood cell count was still only 1.1-1.2 million cells per cubic millimeter. On Nov. 18, 1945, and for each of the following nine days, she was given 20 mg. of synthetic *L. casei* factor intravenously. Her subjective improvement was marked on the third day, the number of reticulocytes had begun to increase by that time and had attained a peak value of 48.2 per cent on the seventh day of therapy. The rise in red blood cells has been dramatic and a level of 3.0 million per cubic millimeter was observed on the fifteenth day (Fig. 4). She then felt very well and was discharged from the hospital. This woman never had a leucopenia. Her platelets did not begin to rise from their initially low level until the ninth day.

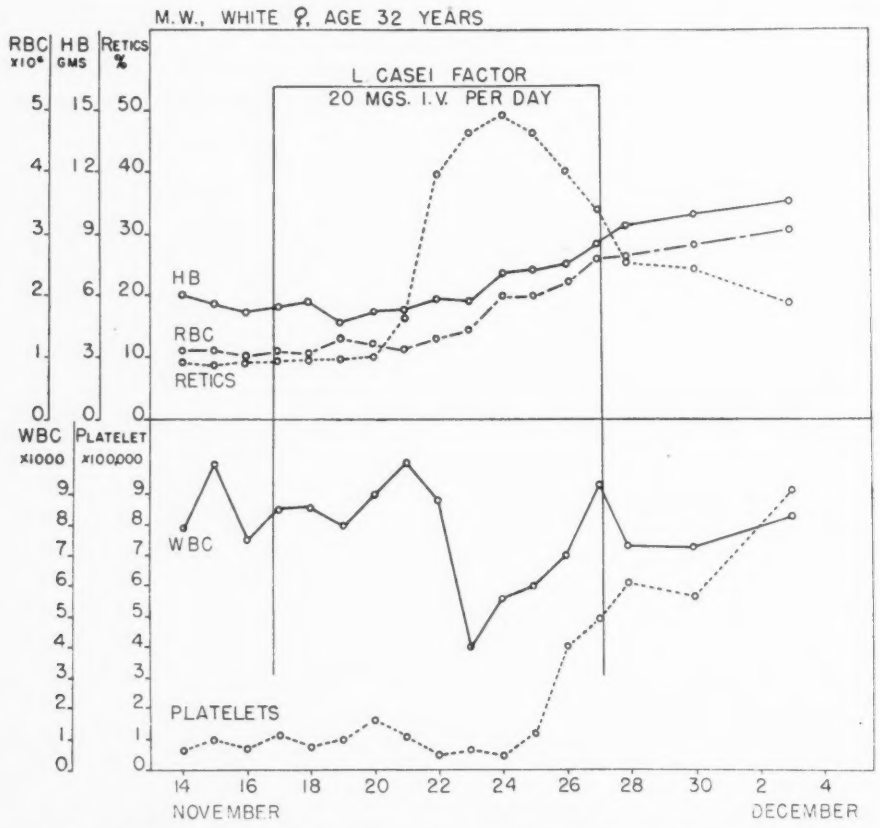

Fig. 4.—Hematologic response of a patient with pernicious anemia of pregnancy to the daily intravenous administration of 20 mg. *L. casei* factor.

The normal control subject, who was given a daily dose of 20 mg. of the material intravenously for ten days, has been followed for three weeks. No change occurred in any of the cellular elements of the blood and no symptoms or exhilaration were experienced.****

DISCUSSION

The fact that synthetic *L. casei* factor, administered orally without gastric juice and intravenously, induced hematologic and clinical remissions in these patients, suggests that the compound has antipernicious anemia rather than extrinsic factor activity. Caution must be used in drawing final conclusions

about this differentiation, however, for two reasons. Wintrobe[17] has shown that good therapeutic responses may be induced in patients with Addisonian pernicious anemia by the daily oral feeding of 1 to 2 grams of brewer's yeast per kilogram of body weight. If yeast contains only extrinsic factor activity, as has been thought, then the small amounts of intrinsic factor present in the gastric secretions of patients with pernicious anemia form enough erythrocyte maturation factor to be therapeutically effective. The daily doses of 30 mg. or of 100 mg. of synthetic *L. casei* factor may possibly be comparable in extrinsic factor activity to the large doses of yeast used by Wintrobe. Second, no extrinsic factor preparation has ever been available in pure enough form to permit its use parenterally. There is, therefore, no information available as to what effect Castle's extrinsic factor would have were it to be injected intravenously.

Even if synthetic *L. casei* factor has antipernicious anemia factor activity, as the available evidence seems to indicate, it has not been established that this factor is identical with the antipernicious anemia factor in liver extracts. The parenteral doses used were 20 mg. daily; investigators who have fractionated liver extracts in an attempt to isolate the active principle have obtained good responses with much smaller quantities of material. Strandell, Poulsson, and Schartum-Hansen,[18] for instance, produced a good hematologic effect with 3 injections of a fraction which contained only 0.7 mg. of material per injection. Alexander[19] was able to obtain a reticulocytosis of 11.4 per cent in a patient with pernicious anemia, whose original red blood cell level was 1.9 million cells per cubic millimeter, when 0.035 mg. of a fraction was given per day. Furthermore, Wright and Welch[12c] found that a commercial liver extract (Armour; 15 U.S.P. injectable antipernicious anemia units per cubic centimeter) contained only 14 mg.-units of microbiologically active "folic acid" (*L. casei* assay) per cubic centimeter, or about 0.25 to 0.5 micrograms of *L. casei* factor per unit of antipernicious anemia activity. Clark[20] has reported that various commercial liver extracts contain, per U.S.P. unit, only 0.02 to 3.67 micrograms of *L. casei* factor. A liver extract made by the Wilson Laboratories (2 U.S.P. units per cubic centimeter) has only 0.38 micrograms of "folic acid" per unit.[21] In terms of this latter preparation, the intramuscular administration of 1 U.S.P. unit daily for 10 days would provide only 3.8 micrograms; with the 20 mg. dose of synthetic *L. casei* factor, we actually gave 200,000 micrograms during a similar period. Although the amounts of conjugated or "potential" *L. casei* factor in these liver extracts are not known, it is evident that synthetic *L. casei* factor must be proved effective in much smaller doses before it can be accepted as being identical with the antipernicious anemia factor in liver.****

Rat liver has been found to contain only about 10 micrograms or less of free *L. casei* factor per gram.[22] The daily ingestion of 250 to 300 grams of pork or beef liver will usually provide a satisfactory therapeutic effect for patients with Addisonian pernicious anemia. Since pork and beef liver contain similar amounts of microbiologically active "folic acid," only 2.5 to 3.0 mg. of free "folic acid" would be supplied by 250 to 300 grams. Under these circumstances, one would expect that if the *L. casei* factor were the antipernicious anemia factor present in liver, the patient who received 100 mg. of the synthetic factor daily for ten days should have had enough active principle to raise her red cell level to 5 million rather than to only 3 million cells. It may be, as SubbaRow, Hastings, and Elkin[23] suggest, "that the activity of material effec-

tive in the treatment of pernicious anemia rests upon a certain type of compound or linkage common to more than one substance, as is believed to be the case of some of the vitamins."

On the other hand, the data available on the "folic acid" content of various tissues and foods have been obtained through the use of microbiological assays and it is now known that many materials contain relatively large amounts of *L. casei* factor in a combined form (milk: Welch and Wright,[24] Wright and co-workers[25]; yeast: Binkley and associates,[26] Mims, Totter, and Day[27]). Mallory, Mims, Totter, and Day[28] and Binkley and his co-workers[26] have studied the enzymatic release of microbiologically active material from the combined or conjugated form, and Laskowski and associates[29] have partially purified an enzyme found in chicken pancreas (and other tissues) which accomplishes this release of the free *L. casei* factor. It is not unlikely that the amount of "potential" *L. casei* factor ingested in the diet is very much larger than microbiological assays have indicated. It is conceivable that in certain pathologic conditions there is a failure to utilize or to absorb this "potential" *L. casei* factor, as a result of which anemia of the macrocytic type might develop. Possibly the intrinsic factor of Castle is concerned with the release in man of the *L. casei* factor from its combined form.

The significance of and the variations in the synthesis of the *L. casei* factor by the intestinal bacteria, so extensively studied in the rat, must now for obvious reasons be thoroughly investigated in man.

Since the patients reported by Spies and his co-workers[2] were said only to have macrocytic anemia in relapse, and no differentiation was made between nutritional macrocytic anemia and Addisonian pernicious anemia, the two patients with true pernicious anemia here reported are the only two so far known to have responded specifically to synthetic *L. casei* factor. The woman with pernicious anemia of pregnancy is also of special interest. Patients with this disease usually respond to parenterally administered liver extract, but Watson and Castle[30] have recently observed two such patients who responded to oral but not parenteral therapy. This observation raised the question as to whether the pathogenesis of pernicious anemia of pregnancy is always the same as that of Addisonian pernicious anemia. It is of interest, therefore, that *L. casei* factor administered parenterally was capable of inducing a remission in at least this one subject.

Finally it should be pointed out that in none of the cases so far studied has the synthetic *L. casei* factor been given for a sufficiently long period to determine whether relapses will occur during continued administration of the vitamin. There has not been sufficient time to determine what effect *L. casei* factor has on the neurologic manifestations of pernicious anemia.

SUMMARY AND CONCLUSIONS

Clinical and hematologic remissions were induced in two patients with Addisonian pernicious anemia by the daily oral administration for ten days of 30 mg. and of 100 mg. of synthetic *L. casei* factor ("folic acid"), respectively. One patient with macrocytic anemia of nontropical sprue and one patient with pernicious anemia of pregnancy responded in a similar manner when given daily 20 mg. of the preparation intravenously.

Reasons are given for interpreting these results as indicating that *L. casei* factor possesses antipernicious anemia factor activity. It is pointed out, how-

ever, that the material must be proved effective parenterally in much smaller doses, and over a much longer period of time, before it can be accepted as being closely similar in action to (or identical with) the effective principle present in liver extracts.

CASE REPORTS

M. G., white female, 63 years of age. This woman was admitted to the St. Louis City Hospital on Oct. 24, 1945 with the chief complaints of progressive pallor, weakness, and weight loss during the previous three months. She had been a ward attendant at the City Hospital for many years. During the summer, she noticed that the work she previously had done without difficulty was becoming increasingly hard. As a result, she decided not to return to her job after her vacation in August. Even with very little work to do at home, however, she was increasingly aware of her weakness and her family observed that she was becoming pale. There was a weight loss of approximately 13 pounds from midsummer to October. Five days before admission she had an emesis of green-colored vomitus which contained no food particles, she felt dizzy, and had a sense of substernal oppression. The latter symptom recurred whenever she moved about her home and disappeared within a few minutes after she would lie down. Melena had never been observed. She did not complain of paresthesias.

The patient has been in reasonably good health during most of her life. Her hair began to turn white at the age of 55 years. Her only previous hospitalization had been for an upper respiratory infection in February, 1945; at that time her red blood cell count was normal.

Physical examination showed the patient to be a rather small, blue-eyed, white-haired elderly woman who lay very quietly in bed and slept almost constantly except when she was disturbed. Her skin and mucous membranes were pale and a distinct lemon yellow color was present. Vessels of her retina showed arteriosclerotic changes. She was edentulous and the sides of her tongue were smooth. There was a dorsal kyphosis and fine moist rales were heard at both bases. The area of cardiac dullness extended 10 cm. to the left of the midsternal line and a soft systolic murmur, loudest at the base, was heard over the whole precordium. Cardiac rhythm was made irregular by frequent premature systolic contractions. Blood pressure 120/52. Liver and spleen were not palpated; no abdominal masses were felt. Pelvic and rectal examinations were essentially negative. There was no significant degree of lymph node enlargement. A few small purpuric areas were present over the dorsum of the hands and forearms. All deep reflexes were normally active and of equal intensity on the two sides. There was no detectable disturbance of position or of vibratory sense.

Urinalysis was within normal limits. No occult blood or parasites were found in the feces. The Kahn test was negative. The initial blood studies showed: red blood cell count 1,170,000 cells per cu. mm., hemoglobin 5.4 Gm., reticulocytes 0.7 per cent, hematocrit 18 per cent, white blood cell count 3,400. There was marked anisocytosis and poikilocytosis of the red blood cells with a mean corpuscular volume of 158 cubic microns and a mean corpuscular hemoglobin concentration of 33 per cent. Leucocytic differential was within normal limits but the polymorphonuclear leucocytes were hypersegmented. Bone marrow removed by sternal aspiration appeared hyperplastic, particularly for erythroid elements. There was a distinct increase in the percentage of early erythroblasts and megaloblasts. Icterus index 10. Cephalin cholesterol flocculation test two plus. The I.V. hippuric acid test resulted in a urinary excretion of 0.43 Gm. of sodium benzoate within 1 hour. N.P.N. was 22 mg. per cent. Total plasma protein concentration was 6.88 Gm. per 100 c.c. with 4.43 Gm. of albumin and 2.45 Gm. of globulin. A histamine refractory achlorhydria was present.

Roentgenogram of the chest showed evidence of marked peribronchial fibrosis in both lung fields, areas of calcification in the descending aorta, moderate cardiac enlargement, and kyphosis in the midthoracic region. Fluoroscopic study of the gastrointestinal tract revealed no abnormalities except two rounded areas of irregular calcification in the gall bladder region. Electrocardiogram showed the changes of left bundle branch block.

As is stated in the body of the paper, this patient experienced a striking sense of well-being on the third day of therapy with *L. casei* factor. She has now improved markedly in strength, is gaining weight, and no longer experiences substernal oppression when she walks around the ward.

E. K., white female, 73 years of age. This woman is a spinster who lives alone. The history she gives is not entirely coherent and other members of the family are not able to provide much additional information. Her hair had begun to turn white before she was 30 years old. Menopause occurred ten years later. In 1927 or 1928, she noted weakness, tingling in her feet and hands, sore tongue, dyspnea, and slight ankle edema. The physician consulted told her she had an anemia and advised her to eat liver. She improved remarkably and all symptoms other than her paresthesias disappeared. A number of doctors were seen between this time and 1939; some gave her liver extracts but the patient was quite irregular either in reporting to her physicians or in eating liver. In October of 1939, she became nauseated and vomited persistently for several weeks. She became increasingly weak and was soon confined to bed. About the middle of November her sister told her she was jaundiced. There was no hematemesis, no melena. A weight loss of 10 to 20 pounds occurred during this period.

On Nov. 21, 1939, the patient was admitted to the St. Louis City Hospital. She was pale, edentulous; had a smooth, glistening tongue; her liver was palpable 2 cm. below the right costal margin; pitting edema of both feet and legs was detected; the Babinski sign was bilaterally present. Blood pressure 140/60. Her red blood cell count was 1,200,000 cells per cubic millimeter, hemoglobin 5 gm., and white blood cells 4,500. The red cells were macrocytic and varied greatly in size and shape. Bone marrow studies were said to be compatible with a diagnosis of Addisonian pernicious anemia. A histamine refractory achlorhydria was present. Twenty units of liver extract were given parenterally each day. By the seventh day, her reticulocytes had risen to 35 per cent. When she was discharged from the hospital, the red blood cell level had risen to 4,080,000 and the hemoglobin to 14.5 grams. No other therapy had been given. Fluoroscopic study of the gastrointestinal tract revealed no abnormalities.

For several years, the patient returned at regular intervals to the Out-patient Department for injections of liver extract. She felt well and was unusually agile for a woman of her age. In May of 1941, she was readmitted to the City Hospital for several days because of a skin eruption on her legs which was said to be an "eczema." It disappeared after boric ointment was regularly applied. Her red blood cell count at that time was slightly over 4 million cells.

Visits to the Clinic became irregular in 1944 and finally stopped altogether about five months previous to the present hospitalization. She remained well until the end of October when she again became nauseated and began to vomit. She lost her appetite, became progressively weaker, and became bedfast early in November. There was no orthopnea, no dyspnea, but ankles were occasionally swollen. No hematemesis or melena was observed.

Physical examination revealed a lethargic, almost comatose elderly woman who was very pale and who had a slight icteric tint to her sclera and skin. Her temperature was 38.5° C., pulse 92, respirations 36, and blood pressure 120/50 mm. of Hg. Her skin was dry and hot. Tongue was smooth; mouth, edentulous. There were signs of fluid in the right pleural cavity. A moderately loud systolic murmur was audible over the entire precordium. No masses or organs were palpable in her abdomen. Reflexes were normally active; there were no pathologic toe signs; vibratory sense was diminished over both legs.

Urinalysis showed 2 plus protein and a rare granular cast. The hematologic data are summarized on the chart (Fig. 2). Red blood cells were macrocytic and varied greatly in size and shape. The differential white blood cell count was normal except for an occasional myelocyte and for hypersegmentation of the polymorphonuclear neutrophils. Sternal marrow was hyperplastic and showed a striking increase in megaloblasts. Blood Kahn test was negative; the N.P.N. was 32 mg. per cent. Total plasma proteins were 5.2 Gm. per 100 c.c. with 3.3 Gm. of albumin and 1.0 Gm. of globulin. Venous pressure was 122 mm. of saline. Pleural fluid showed a specific gravity of 1.013 and a protein content of 2.85 Gm. per 100 cubic centimeters.

Because of the patient's critical condition, a transfusion of 500 c.c. of citrated blood was given shortly after she entered the hospital. Her temperature returned to normal. The following day administration of 30 mg. *L. casei* factor orally per day was begun. On the sixth day, however, signs of bronchopneumonia developed and a decubitus ulcer formed over her sacral area. She was given 1.0 mg. digoxin intravenously. Administration of penicillin in doses of 40,000 units every 2 hours (intramuscularly) was begun; after 12 hours, the amount was reduced to 20,000 units every 2 hours. Within 72 hours she was markedly improved. Administration of penicillin has now been discontinued. She is eating well, and feels much stronger.

J. S., white female, 54 years of age. During the last four or five years, this woman has had from one to three formed or semiformed, light-colored stools per day. To her knowledge, they have never been foamy. She lost approximately 40 pounds in weight during this period and became progressively weaker. Early in 1945 she noted stiffness of her muscles and generalized pains throughout her body. Shortly thereafter her ankles began to swell but there was no associated dyspnea or orthopnea. She was a patient in the Barnes Hospital from July 2, 1945, to August 24, 1945. At this time it was discovered that she had a hypochromic microcytic anemia with a red cell count of slightly over 4 million cells and a hemoglobin value of approximately 9 grams. Her serum calcium was found to be 7 mg. per 100 c.c., serum phosphorus 3.5 mg. per cent and the alkaline phosphatase 14 Bodanski units. Because of these changes, a diagnosis of osteomalacia was made and she was given large doses of both vitamin D and of calcium. After her discharge from the hospital, she improved steadily but gained very little weight. Since no cause for the development of osteomalacia had been determined, and since it was felt that her improvement had not been satisfactory, she was readmitted to the hospital on Oct. 26, 1945.

Physical examination showed the patient to be an extremely emaciated white woman of middle age who walked slowly with a portable walker. She was comfortable, cooperative, and complained chiefly of the fact that she had been returned to the hospital. Her mucous membranes were moderately pale and her skin showed a slight brownish pigmentation very much like that of a mild sunburn; it was dry and inelastic. There was no significant enlargement of lymph nodes. The tongue was moderately smooth. The heart and lungs were not remarkable. The abdominal wall was very thin so that peristoltic patterns were easily seen; the liver edge was palpable 1 cm. below the right costal margin; no other organs or masses were found. Pelvic examination showed the vaginal mucosa to be atrophic. Rectal examination was negative. Examination of the extremities showed pronounced atrophy of both leg and thigh muscles. Deep reflexes were normally active and equal on the two sides. There were no pathological toe signs and no sensory disturbances were detected.

Urinalysis showed a trace of protein. Stool examinations were repeatedly negative for the presence of occult blood or of parasites. Blood Kahn reaction was negative. Hematologic studies were as follows: red blood cells 2,630,000, hemoglobin 9 grams, reticulocytes 1.2 per cent, white cells 2,850, platelets 129,000. The leucocytic differential was normal. There was definite macrocytosis of the red cells (mean corpuscular volume 114 cubic microns) with anisocytosis and poikilocytosis; the polymorphonuclear neutrophils showed hypersegmentation. Bone marrow removed by sternal aspiration was normally cellular but showed an increase in the percentage of erythroid elements with a definite increase in the number of early erythroblasts and megaloblasts. Total serum protein was 5.9 Gm. per 100 c.c. with 4.0 Gm. of albumin and 1.9 Gm. of globulin. In an oral glucose tolerance test the fasting blood sugar was 69 mg. per cent while the highest value reached following the injection of glucose was only 80 mg. per cent. An intravenous glucose tolerance test the following day showed a fasting blood sugar of 67 mg. per cent; 251 mg. per cent at the end of one-half hour after injection of the glucose; 150 mg. per cent at the end of two hours and a value of 67 mg. per cent at the end of three hours. Gastric analysis showed no free acid after histamine. Five determinations of the amount of fat per dry weight of stool varied from 50 to 76.5 per cent. Serum calcium was 6.8 mg. per cent, serum phosphorus 2.9 mg. per cent and phosphatase 13 Bodansky units. Fluoroscopic studies of the gastrointestinal tract revealed no abnormality. Intestinal contents aspirated from the duodenum were normal for amylase and trypsin, but contained only small amounts of lipase activity.

Since Nov. 5, 1945, this woman has been given an average of 20 mg. of synthetic *L. casei* factor intravenously per day. Her reticulocytes began to increase on the third day and reached a peak value of slightly over 30.2 per cent. The red cell count has risen from approximately 2.5 to approximately 3.5 million cells, and the erythrocytes have now become hypochromic. The patient is much improved clinically, feels stronger, and has gained six pounds in weight.

M. W., white female, 32 years of age. This woman had always been in reasonably good health until the last trimester of her first pregnancy in 1935. At that time, and during the last two months of each of her four subsequent pregnancies, she became weak, pale and moderately short of breath. Each of her children was delivered in a different hospital and each obstetrician told her that she had a severe degree of anemia. Transfusions were given at the time her third and fourth children were born; liver extract was also administered but no careful hematologic study was made of her response to therapy. She was admitted to the St. Louis Maternity Hospital on Oct. 25, 1945, and shortly thereafter delivered her fifth child. It was found that she had a red cell count of slightly over 1 million cells and a diagnosis of

pernicious anemia of pregnancy was made. The patient and her husband, however, refused to let transfusions be given and the patient discharged herself from the hospital when an attempt was made to give her liver extract. On Nov. 14, 1945, she asked to be admitted to the St. Louis City Hospital because she was extremely weak and had by that time become convinced that further therapy was necessary. It is noteworthy that although this woman ate liver about twice a month, she ate only small amounts of any other form of meat. She regularly prepared a meat dish for her family each day, but usually chose to eat none herself. She did regularly drink three glasses of milk a day, eat one or two eggs a day, and usually some cheese. Her weight gain during this last pregnancy was normal.

Physical examination showed the patient to be listless, extremely weak, and very pale. There was a definite lemon yellow tint to her sclera and skin. No significant enlargement of lymph nodes was detected. The papillae of her tongue were not atrophic. Both lung fields were clear; the heart was not enlarged in size; rhythm was regular; a soft systolic murmur was audible over the whole precordium. Liver and spleen were not felt. Pelvic examination showed that the uterus was apparently involuting in a normal manner. Examination of the extremities revealed no abnormality. Reflexes were normally active and equal on the two sides; no sensory disturbances were detected.

Urinalysis was within normal limits. Hematologic study showed the red cell count to be 1,130,000, hemoglobin 5.5 grams, reticulocytes 7.4 per cent, white cells 10,000, platelets 89,300. Leucocytic differential was as follows: myelocytes 3, metamyelocytes 2, stabs 2, segmented neutrophils 53, lymphocytes 36, monocytes 4. There was marked anisocytosis and poikilocytosis of the red cells with definite macrocytosis. Three normoblasts were present for one hundred white blood cells. The hematocrit value was 16 per cent, the mean corpuscular volume 145 cubic microns, and the mean corpuscular hemoglobin concentration 36 per cent. Bone marrow removed by sternal aspiration was very cellular and was characterized both by an increase in the percentage of erythroid elements and a definite predominance of early erythroblasts and megaloblasts. The icterus index was 12 and the serum iron value 0.183 mg. per cent. Blood Kahn reaction was negative. A histamine refractory achlorhydria was present on Nov. 16, 1945, but 11 degrees of free acid were found thirty minutes after histamine on Nov. 27, 1945.

Daily administration of 20 mg. of *L. casei* factor intravenously was begun on Nov. 17, 1945. Within forty-eight hours, the patient experienced a sense of well-being which was quite remarkable. Her red cell count has risen rapidly so that a level of 3 million cells was attained on the fifteenth day after therapy was instituted. She has now been discharged from the hospital.

NOTES

*Fermentation factor of Hutchings and associates.[7b]

**Generously supplied by the Cerophyl Laboratories, through the courtesy of Dr. W. R. Graham, Jr.

***Charcoal adsorption and elution, followed by superfiltrol adsorption and elution. This material had an activity of 1.6 mg. potency 40,000 units per gram; assayed with *L. casei*. As nearly as can be estimated at present, 1 mg. of potency 40,000 units is equivalent to from 0.25 to 0.5 mg. of the pure *L. casei* factor.

****Two additional patients with Addisonian pernicious anemia are currently being given 2 mg. of synthetic *L. casei* factor intravenously per day. Reticulocytes began to rise in both patients on the fifth day of therapy; but the response is submaximal and the clinical improvement has not been dramatic.

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LABORATORY METHODS

A NOTE ON GASTRIC RETENTION IN ONE-HOUR, TWO-DOSE GLUCOSE TOLERANCE TESTS

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ONE of the most popular glucose tolerance tests at the present time is the one-hour, two-dose test devised by Exton and Rose in 1934.[1] The test as originally described is carried out by giving two 50 Gm. doses of glucose thirty minutes apart and determining the blood sugar at 0, 30, and 60 minutes. The criteria of a normal response include a fasting blood sugar within the normal limits of the blood sugar method employed, a rise in blood sugar which does not exceed 75 mg. in the 30-minute sample, a blood sugar level in the 60-minute sample which is less, the same, or does not exceed the 30-minute sample by more than 5 mg., and urine samples that are all negative to Benedict's test. The test is based on Allen's paradoxical law[2] which states that there is no limit of tolerance to glucose given by mouth to the normal animal but that diabetic animals exhibit definite limits of tolerance. The test also utilizes the phenomenon observed by Hamman and Hirschmann,[3] that after successive doses of dextrose given by mouth, the level of each successive rise in the blood sugar curve is lower than the preceding one. Since the original description of the one-hour, two-dose test, there have been several clinical studies made employing the test and criteria as originally described or using some modified criteria for the diagnosis of diabetes mellitus and other disorders of carbohydrate metabolism.[4-9] This report describes studies of the gastric retention of glucose following the one-hour, two-dose test.

Glucose tolerance tests were carried out in sixteen normal male medical students following the procedure of Exton and Rose.[1] After taking the 60-minute blood sample, the gastric contents were withdrawn by a stomach tube and analyzed for glucose. The time required for obtaining the gastric contents was generally about ten to twenty minutes. The venous blood sugar levels determined by the Myers-Bailey method[7] averaged 96, 143, and 139 mg. per 100 c.c. for the 0-, 30-, and 60-minute samples, respectively. However, the amount of glucose recovered from the stomach varied from 38 to 62 Gm. and averaged 50 Gm. There was no correlation between the blood sugar changes in the various subjects and the amount of glucose remaining in the stomach. It is apparent that although 100 Gm. of glucose were ingested in the two doses, less than 50 Gm. of sugar usually emptied from the stomach into the duodenum and correspondingly less than 50 Gm. were absorbed into the blood stream during the hour. This is consistent with our observations and those of others,[4-6, 8, 9] that the average blood sugar pattern is quite similar during the first hour, when either one dose or two doses of glucose are given. Many investigators have failed to give this fact proper attention and have been inclined to accept the two-dose test as a more accurate index of disturbed carbohydrate metabolism.

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Received for publication, May 17, 1945.

The finding of more than 50 Gm. of sugar in the stomach at the end of one hour in the one-hour, two-dose test raises a serious question as to whether the administration of a second dose of sugar is of any advantage in glucose tolerance tests of the Exton-Rose type. Any superiority of the two-dose test over a one-dose test could be merely due to the more critical criteria that are applied to the two-dose test.

SUMMARY

In sixteen normal adults subjected to a one-hour, two-dose glucose tolerance test (Exton-Rose type), over one-half of the administered sugar remained in the stomach at the end of one hour. A question of any advantage in administering two doses of sugar in such tests is raised.

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PHOTOELECTRIC DETERMINATION OF BLOOD THIOCYANATES WITHOUT PRECIPITATION OF PROTEINS

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IN THE treatment of hypertension with potassium thiocyanate, frequent determinations of the amount of thiocyanate in the blood are important to maintain adequate therapeutic levels and to prevent possible toxic effects from overdosage. The colorimetric determination of thiocyanate has been based upon its reaction with a ferric salt to form a colored compound. The original method adapted by Schreiber[1] from the procedure of Leared[2] has been variously modified. One of the most widely used methods is that of Barker,[3] and modifications have been proposed by Crandall and Anderson,[4] Lavietes, Bourdillon, and Klinghoffer,[5] Griffith and Lindauer,[6] Ravin,[7] and others.[8, 9] Molenaar and Roller[10] proposed a photometric method and Ginsburg and Benotti[11] have devised a method using the Evelyn photoelectric colorimeter which does not require the preliminary precipitation of the serum proteins. The method to be described was devised for use with the Klett-Summerson photoelectric colorimeter but

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Received for publication, Aug. 3, 1945.

could easily be adapted for use with photoelectric instruments of other types. The advantages of this method and its accuracy as compared with previous methods will be discussed.

METHOD

Reagents.—

1. Thiocyanate standard*
   (a) Stock—Dissolve 2.0 Gm. of C.P. KCNS in 1 liter of distilled water. Titrate this solution against 20 c.c. of a solution of C.P. $AgNO_3$ (2.924 Gm./liter) plus 5.0 c.c. of concentrated HNO_3, using 1.0 c.c. of a saturated aqueous solution of ferric ammonium sulfate as an indicator. Calculate the dilution of the thiocyanate solution needed to make 20 c.c. of it equivalent to 20 c.c. of the silver nitrate solution. After making this dilution, check by another titration to be sure the potassium thiocyanate solution is exactly equivalent to the silver nitrate solution. This final stock solution contains 100 mg. of CNS ion per 100 c.c.
   (b) Working standard—Dilute 10 c.c. of the stock standard to 100 c.c. with distilled water. This solution contains 10 mg. of CNS ion per 100 c.c.
2. Ferric nitrate solution
   Dissolve 50 Gm. of crystalline ferric nitrate in 500 c.c. of distilled water, add 25 c.c. of concentrated HNO_3 and make to 1 liter with distilled water.
3. Blank solution
   Dilute 25 c.c. of concentrated HNO_3 to 1 liter with distilled water.

Calibration.—In Klett-Summerson tubes mix 1.0 c.c. of working thiocyanate standard, 8.0 c.c. of distilled water, and 1.0 c.c. of ferric nitrate solution. Make this determination in triplicate, read after five minutes against a distilled water blank at zero, using a No. 54 filter (spectral range, 500-570 millimicrons), and record the average of the three readings as the *standard reading S*. Since the reagents are stable, a new standard reading need be determined only at intervals of several months or when a new set of reagents is made up. It will be shown in the subsequent discussion that there is direct proportionality between the readings with the Klett-Summerson instrument and varying concentrations of thiocyanate.

TEST

UNKNOWN	BLANK
4.0 c.c. distilled water	4.0 c.c. distilled water
0.5 c.c. serum	0.5 c.c. serum
Mix.	Mix.
0.5 c.c. ferric nitrate reagent, slowly with shaking	0.5 c.c. blank solution, slowly with shaking

Mix well, let stand five minutes and read in the instrument using the No. 54 filter.

Calculations.—Subtract the reading of the blank from the reading of the unknown to obtain the true reading of the unknown (U).

$$\frac{U}{S} \times 10 = \text{mg. of CNS ion per 100 c.c. of serum}$$

*In the method described by Barker[5] there is an error which apparently has escaped detection by later writers.[6] According to Barker's directions, one dissolves about 1 Gm. of potassium thiocyanate in 800 c.c. of distilled water and then standardizes this against a solution of silver nitrate containing 2.9195 Gm. per liter, so that the final stock solution of thiocyanate contains 100 mg. of thiocyanate ion per 100 c.c. Obviously, a solution of potassium thiocyanate containing 1 Gm. per 800 c.c. is too weak to be made equivalent to the silver nitrate solution described and from such a solution it would not be possible to prepare a stock standard of the required strength.

DISCUSSION

Since potassium thiocyanate is hygroscopic, an accurate standard cannot be prepared by weighing out the salt. Instead, it is necessary to make a solution stronger than the one required and to standardize this against a silver nitrate solution.

The calibration of the Klett-Summerson instrument having a special scale is simplified by the fact that the scale readings are directly proportional to the thiocyanate in varying strengths of standard. The calibration curve is a straight line except for concentrations approaching 20 mg. per cent as is shown in Fig. 1. Therefore, the use of a standard reading S obtained from readings on a standard solution containing 10 mg. of CNS ion per 100 c.c. is valid within the range of values usually encountered in blood thiocyanate determinations.

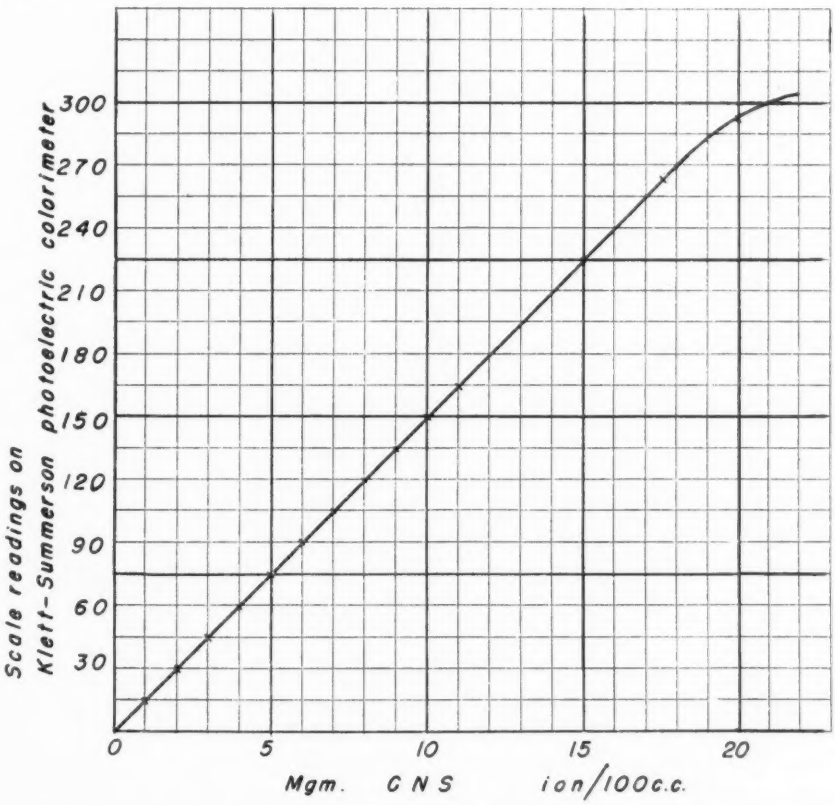

Fig. 1.—Calibration curve for Klett-Summerson photoelectric colorimeter with varying concentrations of thiocyanate standard.

Ginsburg and Benotti[11] employed as a ferric salt a solution of ferric chloride instead of ferric nitrate, since they found that in the proportions of one part of serum to two parts of reagent excessive turbidity was produced by the ferric nitrate reagent. With the method proposed in the present paper, this objection does not apply since the final dilution of serum is 1:10. Numerous observations have shown that any turbidity produced is minimal, is not progressive for periods up to one hour, and is, in addition, perfectly compensated for by the blank determination. The ferric chloride reagent has several disadvantages: First, The reagent itself has a distinct yellow color which precludes the use of a blank reading, except, as is proposed by Ginsburg and Benotti,[11] on serum

from each patient before any thiocyanate is administered. Not only would this procedure be time consuming and often not feasible in routine clinical laboratory practice, but, in addition, it disregards the thiocyanate normally present in human blood serum which is often not inconsiderable. A second disadvantage in the omission of a routine blank determination is that in jaundiced or hemolyzed blood a considerable error is introduced. In the proposed method neither jaundice nor hemolysis alters the accuracy of the final readings since the blank reading compensates for any altered serum color (see Table I).

TABLE I.—EXPERIMENTAL RECOVERY OF THIOCYANATE ADDED TO SERUM

SAMPLE	ANTICIPATED RECOVERY (MG.)	ACTUAL RECOVERY (MG.)	PERCENTAGE RECOVERY (PER CENT)	PERCENTAGE ERROR (PER CENT)
1	2.95	3.0	101.0	+1.0
2 (Red from hemolysis)	5.15	5.10	99.0	−1.0
3	5.3	5.3	100.0	0.0
4 (Red from hemolysis)	5.35	5.35	100.0	−0.0
5 (Red from hemolysis)	6.90	6.93	100.3	+0.3
6	10.23	10.33	100.9	+0.9
7	10.5	10 33	98.3	−1.7
8 (Red from hemolysis)	10.53	10.7	101.6	+1.6
9 (Bilirubin, 3.4 mg. %)	10.53	10.7	101.6	+1.6
10	13.6	13.6	100.0	0.0
11	15.2	15.0	98.6	−1.4
12	16.5	16.3	98.1	−1.9

Maximum per cent error, 1.9; Average per cent error, ±0.95.

A possible third disadvantage in the use of ferric chloride lies in the rapid fading of the color produced. According to Ginsburg and Benotti,[11] the color fades appreciably after five minutes and any delay in reading causes a considerable error. The ferric nitrate reagent, in contrast, produces a color which I have found to be constant for periods up to several hours. However, in my own experiments with the ferric chloride reagent I have had no difficulty with fading but have found the color produced to remain constant for at least one hour.

The accuracy of the method proposed is shown by the quantitative recovery of thiocyanate added to serum in varying amounts as is shown in Table I. Neither jaundice nor hemolysis interferes with accurate recovery.

Trichloracetic acid has been employed as the precipitant in the majority of methods involving preliminary protein precipitation. Chesley[12] concluded, after a number of experiments, that either trichloracetic or tungstic acid was satisfactory. Parallel determinations by the proposed method and a method

TABLE II. COMPARATIVE RECOVERIES OF THIOCYANATE WITH AND WITHOUT PRECIPITATION OF PROTEINS

SAMPLE	ANTICIPATED RECOVERY (MG.)	RECOVERY BY PROPOSED METHOD (MG.)	% RECOVERY BY PROPOSED METHOD (PER CENT)	RECOVERY ON TRICHLOR-ACETIC ACID FILTRATE (MG.)	% RECOVERY ON TRICHLOR-ACETIC ACID FILTRATE (PER CENT)
1	5.0	4.95	99.0	5.0	100.0
2	10.7	10.65	99.0	10.37	96.9
3	12.03	11.85	98.5	11.85	98.5
4	13.02	12.74	97.8	12.60	96.5
5	13.8	14 07	101.9	13.3	96.4
6	14.4	14.4	100.0	13.7	95.1

using a trichloracetic acid filtrate were made on pooled serum containing known, measured amounts of potassium thiocyanate. The results are shown in Table II. The recoveries shown are representative of those recorded for a number of similar experiments and indicate for the proposed method an accuracy equal or even superior to that of methods employing protein-free filtrates.

SUMMARY

1. A rapid, simple method for the photoelectric determination of thiocyanate in serum without precipitation of proteins has been described.

2. Thiocyanate added to serum in known amounts is recovered with an average error of approximately ± 1.0 per cent.

3. The advantages of the proposed method over previous methods have been discussed.

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A COLORIMETRIC DETERMINATION OF PARALDEHYDE

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A METHOD for the determination of paraldehyde in biologic fluids, following its administration, is provided by the p-hydroxybiphenyl color reaction.[1] The recovery experiments herein summarized show that the convenient procedure described by Stotz[2] for the analysis of blood acetaldehyde can be applied with little modification to the determination of paraldehyde.

The color is developed by the following procedure (for additional details, see reference 2): To 1 c.c. of the solution containing between 0.2 and 2.5 μg. paraldehyde, add 0.05 c.c. of 5 per cent copper sulfate; cool in an ice bath while 8 c.c. of concentrated H_2SO_4 (specific gravity, 1.84) are added slowly with agitation. Deliver 0.2 c.c. of the p-hydroxybiphenyl reagent (1 per cent p-hydroxybiphenyl in 0.5 N NaOH) directly into the solution; disperse the precipitate by shaking, and allow the tube to stand for one hour at room temperature with occasional shaking. Then heat in a boiling water bath for one and one-half minutes, cool, and read the intensity of the color in a photoelectric colorimeter with a 565 filter. A reagent blank prepared simultaneously with 1 c.c. water is used to adjust the galvanometer to 100, and the concentration of paraldehyde in the unknown is obtained from a standard curve; the latter is prepared by developing and reading the colors, as previously described, with a series of solutions containing known amounts of paraldehyde.

Possible sources of error in the use of this reagent have been described by Barker and Summerson.[3] The development of a strong green color in the test upon the addition of the p-hydroxybiphenyl reagent results in erratic and inadequate color development and is usually due to the use of impure sulfuric acid. Inadequate cooling of the solution during the addition of the H_2SO_4 may give rise to a lactic acid color reaction.

When the usual 1:10 tungstic acid filtrate of blood is further diluted ten times or more, the color can be developed directly with a 1 c.c. aliquot. The expected blood levels of from 2 to 25 mg. per cent paraldehyde[4, 5] can thus be determined by the analysis of 1 c.c. of a 1:100 blood filtrate, and larger amounts can be determined on more dilute filtrates. Complete recoveries were obtained by this procedure when from 2 to 100 mg. per cent paraldehyde were added to blood; without added paraldehyde, 1:100 blood filtrates gave no color in the test. A 0.1 c.c. sample of finger-tip blood deproteinized in 10 c.c. dilute tungstic acid (prepared from 0.2 c.c. of 10 per cent sodium tungstate and 0.2 c.c. of 2/3 N H_2SO_4) provides a convenient filtrate for paraldehyde analysis.

Less than 2 mg. per cent paraldehyde cannot be determined satisfactorily by a similar analysis of an undiluted 1:10 blood filtrate, since this more concentrated filtrate gives some color in the absence of added paraldehyde. These smaller amounts, as well as higher concentrations, can be determined by the analysis of a 1 c.c. aliquot of a distillate prepared in the manner described by Stotz.[2] The distillation procedure eliminates the interference by other blood

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Received for publication, Sept. 12, 1945.
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constituents, such as lactic acid, and is therefore more reliable than a direct color development on the diluted filtrate. Complete recoveries of from 0.2 to 3 mg. per cent added paraldehyde were obtained in 5 c.c. of distillate when an aliquot of a 1:10 filtrate of blood was diluted to 10 c.c. and distilled. The trace of acetaldehyde normally found in blood[2] is a limiting factor in the analysis of minimal quantities of paraldehyde, and this interference becomes detectable when the paraldehyde concentration is less than 0.5 mg. per cent. Complete recoveries of from 2 to 100 mg. per cent added paraldehyde were obtained in 10 c.c. of distillate when 1 c.c. of the 1:10 tungstic acid filtrate was diluted to 15 c.c. and distilled. Urinary paraldehyde can also be determined by the distillation method; a preliminary precipitation of an aliquot with phosphotungstic acid (25 c.c. of 25 per cent phosphotungstic acid per 100 c.c. urine) and removal of the precipitate avoid excessive foaming during the distillation.

TABLE I. RECOVERY OF PARALDEHYDE ADDED TO BLOOD

PARALDEHYDE ADDED TO 1 C.C. BLOOD (μG)	PER CENT RECOVERY	
	DISTILLATION METHOD	DIRECT METHOD
2	123	--
3.5	110	--
5.5	101	--
7	98	--
15	101	--
20	99	--
25	95	--
30	87	110
70	100	100
150	100	104
200	94	102
300	99	100
400	99	95
500	102	102
750	97	97
1,000	95	98

The paraldehyde was added to the water with which the blood was laked during the preparation of a protein-free filtrate, and the latter was analyzed for paraldehyde by both the distillation and direct methods.

Paraldehyde was found to be quite stable in whole blood or in a tungstic acid filtrate during storage in a stoppered tube at 4° C.; less than 10 per cent loss occurred in three weeks. This method will not distinguish between acetaldehyde and paraldehyde, but the blood level of acetaldehyde is normally from 0.02 to 0.05 mg. per cent and would not be expected to exceed 2 or 3 mg. per cent even in acute alcoholism.

SUMMARY

A procedure is described for the determination of paraldehyde in biologic fluids by means of the p-hydroxybiphenyl color reaction.

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THE J. S. B. STAIN FOR BLOOD PARASITES

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WASHINGTON, D. C.

ALTHOUGH stained blood films are almost universally used for the demonstration of such parasites as the malaria parasites and Trypanosomes, no one method has received general acceptance. That this is true is evidenced by the new techniques, or modifications of old ones, which are continually appearing in print. The chemical reactions involved in the staining of cells are still largely unknown, and staining methods are still largely empirical.

Most of the commonly employed blood stains belong to the Romanowski group, so called because the Russian, Romanowski, devised the parent of them all in 1891. He used a combination of methylene blue and eosin which has been modified in different ways by numerous workers since but fundamentally improved by only a few. Of such modifications, Giemsa's is generally considered the best. Nevertheless, even this method has certain disadvantages, for it is rather slow and the stain is costly and difficult to prepare.

It is probable that in the United States, Wright's is more widely used than Giemsa's method. It gives excellent differentiation, is faster, and is less expensive. However, it is less uniform in the quality of results and does not keep well in hot climates.

In the United Kingdom, Leishman's modification is very popular. It is similar to Wright's in the manner of use and probably is more consistent in the quality of staining. It is, however, very difficult to obtain in the United States, and films stained with Leishman's are said to fade more readily than those stained by Wright's or Giemsa's technique.

Recently a new method has been introduced by Field (1940) which has been widely adopted because of its speed. By its use a thick smear can be stained in as little as ten seconds, and, provided drying can be hastened by placing in an incubator or current of hot air, it is ready for examination very soon afterward. Thus this method has a great advantage for a busy physician or technician who must make his diagnosis as soon as possible. But Field's stain is of little value for thin smears and does not give preparations which in any way equal those obtained by the older processes just mentioned.

This paper concerns a staining method which was devised and very recently reported by Singh and Bhattacharji (1944) and which seems superior to all other Romanowski staining processes wherever results of high quality, quickly obtained, are desired. We have used the method quite extensively and have found it to possess these advantages:

1. It is easily prepared from ordinary medicinal methylene blue and eosin.
2. It is relatively inexpensive.
3. It keeps well under different climatic conditions.
4. It is extremely fast (for thin smears, eighty seconds; thick films, thirty seconds).
5. It is equally good for both thick and thin smears, which may be stained together on the same slide.

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Received for publication, Sept. 28, 1945.
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6. Solutions used for staining do not have to be made up each time, since they deteriorate little on standing.

7. Blood cells and parasites are clearly and brilliantly differentiated.

8. Results depend less on the pH of the diluting agent than with other Romanowski stains (especially Giemsa's).

Since the original article was published in India and may not be available to many workers in this country, directions for the preparation and use of the stain are given here. Certain changes which the originators of the stain have recommended since publication of their first paper have been included.

Two solutions are used. Solution I is made up from the following ingredients:

Medicinal methylene blue	0.5 Gm.
Potassium dichromate	0.5 Gm.
Sulfuric acid (1 per cent)	3.0 c.c.
Water	500.0 c.c.

"Dissolve the methylene blue thoroughly in 500 c.c. of water. Add the 1 per cent sulfuric acid, mix thoroughly, and then add the chrome salt. A heavy amorphous purple colored precipitate of methylene blue chromate forms. Heat in an autoclave at a temperature of 100° to 109° C. and a pressure of 0 to 5 pounds for three hours. At the end of this period, the solution turns blue which indicates almost complete polychroming. If the color remains greenish, further heating for another hour or so is required. If the temperature is allowed to rise above 110° C., the oxidation of methylene blue may be carried too far and the solution will turn a violet purple.

"When the solution has turned deep blue after three hours' boiling, allow it to cool at room temperature. Then add 10 c.c. of 1 per cent potassium or sodium hydroxide solution, drop by drop, very gradually while constantly shaking the flask. After the total amount of alkali has been added, transfer half of the contents of the flask into another of the same capacity and continue shaking for fifteen minutes more. Transfer the contents of the flasks into each other. In this way the precipitate will gradually get dissolved and the solution will turn deep blue with a violet iridescence. Leave it at room temperature for forty-eight hours for the solution to mature; afterward filter through a soft filter paper. . . . The solution will improve in staining qualities with age.

"Solution II. This is readily prepared by dissolving 1 Gm. of water-soluble eosin in 500 c.c. of tap water. A freshly prepared eosin solution may not yield as satisfactory a stain as one which has turned deep red after some use.

"Solutions I and II should be kept in wide-mouth stoppered jars, 1½ inches in diameter by 3½ inches in height, and set aside for forty-eight hours to mature. These keep well for several months, but in Solution I a thin golden yellow scum is likely to form on its surface due to a slight precipitation of the dye. This does not, however, interfere with staining, and the staining power of the solutions does not deteriorate with age."

Directions for using the stain are given as follows:

"Thick and thin smears taken on the same slide can be easily stained.

1. Fix the thin smear by dipping that part of the slide in a jar containing methyl alcohol for a minute or two.

2. Dry thoroughly, preferably by waving the slide in the air.

3. Immerse the whole slide in Solution I for thirty seconds.

4. Wash in a jar containing acidulated tap water (pH 6.2 to 6.6). With tap water in Delhi (pH 7.6, indicator, bromo-thymol-blue) approximately 50 mg. of sodium dihydrogen phosphate or 5 drops of 5 per cent acetic or citric acid solutions for each 100 c.c. of water are necessary.

5. Stain with Solution II for one second.

6. Wash in the same jar (4) for four seconds.

7. Immerse in Solution I again for thirty seconds (3).

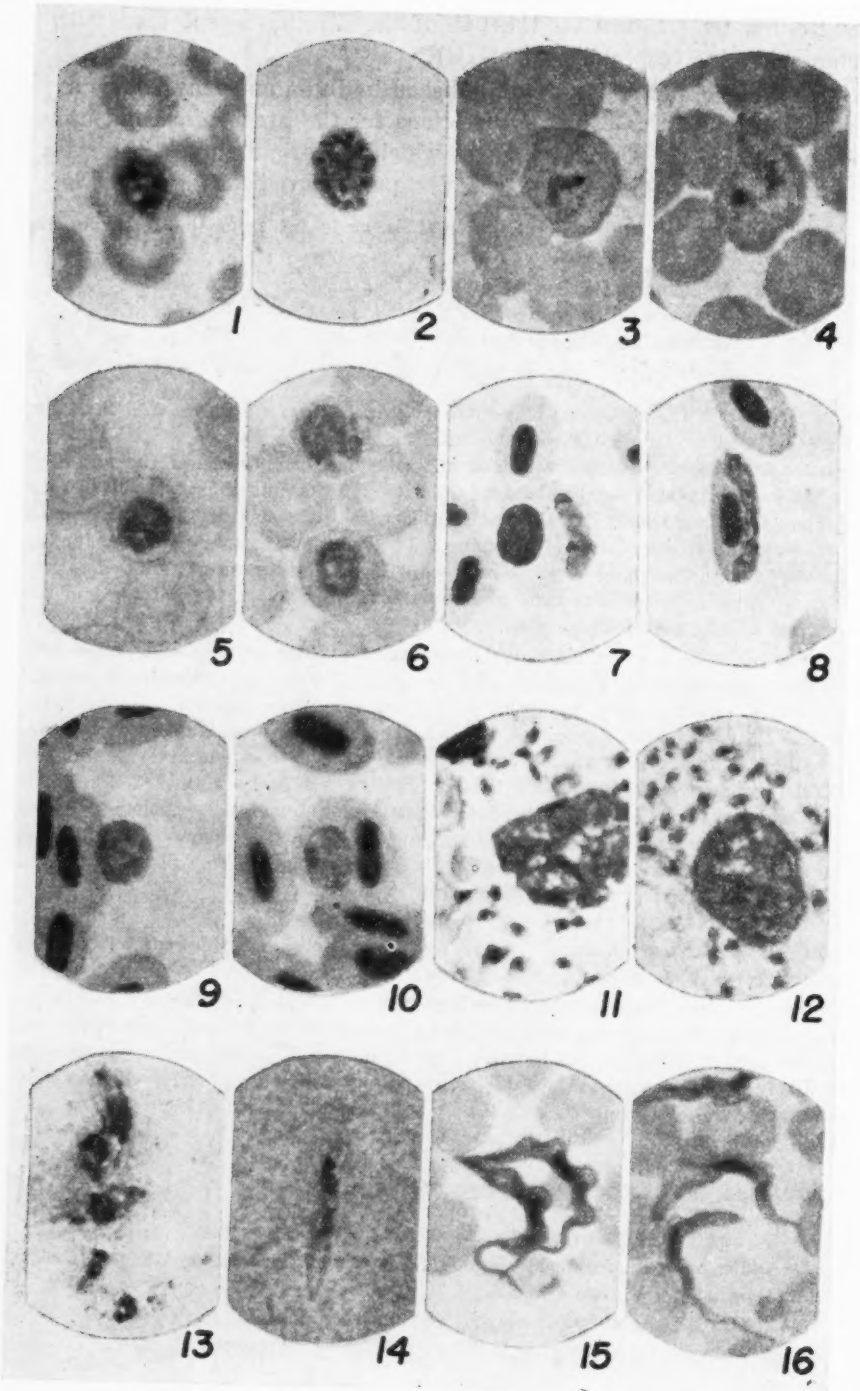

Fig. 1.—*(See opposite page for legend.)*

8. Wash as above for ten seconds or till the smear gives a pink background (4).

9. Dry and examine.

Thick Smear Alone

1. Immerse the slide in Solution I for ten seconds.

2. Wash in a jar containing water acidulated (pH 6.2 to 6.6) for two seconds.

3. Stain with Solution II for one second.

4. Wash in the same jar (2) for five seconds.

5. Immerse in Solution I again for ten seconds.

6. Wash as above for two seconds or till the smear gives a pink background (2).

7. Dry and examine.

The total time taken for staining a combined thick and thin smear is one minute and twenty seconds, and for a thick smear, only thirty seconds.''

We have found that all the common blood parasites for which Giemsa's or Wright's stains are usually used can be demonstrated equally well by the method just outlined. Altering the pH of the medium, and particularly of the water used for washing the slide, appears to make very little difference in the results obtained. However, an acid pH does tend to give the erythrocytes a rather reddish or orange appearance, while a more alkaline reaction results in giving them a grayer or more bluish cast.

Since Giemsa's method seems to give the best results for most of the blood protozoa, it seemed worth while to compare the two methods as exactly as possible. Accordingly, smears were prepared of a number of species of Plasmodia, several species of Trypanosomes, and of *Leishmania donovani*. Some of each parasite were then stained by each method. These were critically compared under the microscope, and a series of photographs was made as shown in Fig. 1. The photographs in the first and third columns were made from slides stained by the Giemsa technique and those in the other two columns show the same species of parasite stained by the J. S. B. process. It will be noted that the two techniques give equal differentiation. Actually the color characteristics of both parasites and blood cells are similar, whichever the process used, but black and white photographs cannot give clear evidence of this fact.

To determine how the two stains compared with respect to fading, slides of *Plasmodium malariae* prepared by each process were divided into four groups. These groups were similar in number, and the slides in each were comparable in every respect. One of the groups served as a control, and the other three were subjected to the radiation from a General Electric sunlamp of 450 watts. The ultraviolet light from this lamp is said to equal five times the ultraviolet

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Fig. 1.—The microphotographs in the first and third columns were stained by the Giemsa technique; the others were stained by the J. S. B. method. In each case comparison is easy, since the same species of parasite is shown stained by each of the two methods. Magnification is ×1360.

1, A schizont of *Plasmodium knowlesi*. Giemsa, at pH 7.4. (AMM negative 90231.) *2*, A segmenter of the same species. J. S. B. at pH 6.5. (AMM negative 90234.) *3*, A ring form of *Plasmodium vivax*. Giemsa at pH 7.4. (AMM negative 90227.) *4*, A slightly larger stage, also vivax. J. S. B. at 6.5 pH. (AMM negative 90228.) *5*, A large trophozoite of *Plasmodium malariae*. Giemsa at pH 7.4. (AMM negative 90262.) *6*, Two parasites (trophozoite and young gametocyte ?) of malariae. J. S. B. at 7.4 pH. (AMM negative 90266.) *7*, Young gametocyte of *Plasmodium elongatum*. Giemsa at pH 7.4. (AMM negative 90240.) *8*, An older macrogametocyte of the same species. J. S. B. at pH 6.5. (AMM negative 90247.) *9*, Macrogametocyte of *Plasmodium relictum*. Giemsa at pH 7.4. (AMM negative 90277.) *10*, *Plasmodium relictum*, probably also a female gametocyte. J. S. B. at 7.4. (AMM negative 90258.) *11*, *Leishmania donovani*, spleen smear (hamster). Giemsa at 7.4. (AMM negative 90249.) *12*, *Leishmania donovani*, also spleen smear. J. S. B. at pH 6.5. (AMM negative 90255.) *13*, *Trypanosoma cruzi* (culture). Giemsa at pH 7.4. (AMM negative 90215.) *14*, *Trypanosoma cruzi* (culture). J. S. B. at pH 7.4. (AMM negative 90210.) *15*, *Trypanosoma equiperdum*. Giemsa at pH 7.4. (AMM negative 90226.) 16, *Trypanosoma equiperdum*. J. S. B. at pH 6.5. (AMM negative 90224.)

radiation of full sunlight at a distance of thirty inches, so that the slides, which were placed only fifteen inches away, received twenty times as much ultraviolet radiation as from the sun in an equivalent time. Group I was exposed 9.3 hours; Group II, 35.4 hours; and Group III, 53 hours. Sunlight equivalents would be 186, 708, and 1,060 hours, respectively.

It was found that some fading occurred in all the radiated groups, and that slides stained with the J. S. B. stain suffered somewhat more than those prepared by Giemsa. Furthermore, slides on which stains buffered with the lower pHs were used faded more than those to which stains of more alkaline pH were applied. It is therefore clear that staining in an alkaline buffer gives more lasting preparations; yet even on those slides which faded the most, parasites and leucocytes were still easily visible, although the reddish components of the dyes had been largely bleached out.

CONCLUSIONS

The J. S. B. stain, recently introduced by Singh and Bhattacharji, has been tested and been found superior in most respects to any of the other commonly used processes for the staining of blood and blood protozoa. The technique is simple and the staining process can be completed in less than two minutes for thin smears and in less than one minute for thick films. The staining solutions are not difficult to make up, are relatively inexpensive, and keep well for weeks or months, even in hot weather. Preparations stained by this process appear very much like those made by Giemsa's method, cytoplasm and chromatin of blood cells and parasites being differentiated with equal clearness and having similar color values. J. S. B. preparations are somewhat less resistant to fading but will stand much more exposure to light than they would ordinarily receive.

Thanks are due T/4 Ruth Cutter Burkenbilt for assistance in this study.

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Erratum

In the article by Andres Goth, entitled "The Antitubercular Activity of Aspergillic Acid and Its Probable Mode of Action," which appeared in the November issue of the JOURNAL, the sentence beginning in the sixth line from the bottom on page 900 should read: "However, these red colonies failed to grow as well as the controls to which no aspergillic acid had been added."

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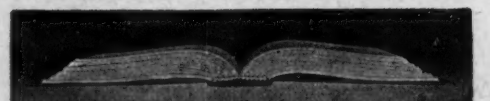

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